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MECHANISM AND EVALUATION OF ANTISEPTICS

BY

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Herbert L. Davis

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MECHANISM AND EVALUATION OF ANTISEPTICS

Consulting Editor and Conference Chairman
Herbert L. Davis

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This series of papers is the result of a Conference on *Mechanism and Evaluation of Antiseptics*, held by the Section of Biology of The New York Academy of Sciences, October 28 and 29, 1949.

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INTRODUCTION

By Herbert L. Davis
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These opening remarks will be quite brief in setting forth some general notions, some of which may deserve no higher designation than "hypotheses," but may suggest broad pictures to guide research and interpretation. Students of colloid chemistry have long felt that most fruitful results would attend the wider application of colloid principles and points of view to biological problems. All living organisms are colloidal structures, and the most important biological substances are proteins and protein complexes. Several lines of evidence indicate that antiseptic substances, whether of natural or synthetic origin, must first be adsorbed on and in the microorganism, and that effectiveness is primarily a function of the extent and firmness of this adsorption.

This adsorption of antiseptics from true solution or from colloidal dispersion will be a resultant of many factors, but, if all the other variables, such as pH, temperature, concentration of organisms, time of exposure, *etc.*, be fixed, it appears that some antiseptics may exhibit four different concentration effects, as shown in FIGURE 1. At low concentrations, there may be activation of growth; then comes a range of partial inhibition (decreased plate counts); and then a range with zero counts on transfer of bacteria-antiseptic to a culture medium. Over a certain range, however, the addition of suitable neutralizer will restore growth, and then beyond some concentration, effective neutralization fails to produce growth. These latter ranges are, respectively, bacteriostatic and bactericidal concentrations. It is recognized that limited solubility or unfavorable solution conditions may prevent some antiseptics from showing the fourth or even the third effect, but a clear recognition of just what is happening is fundamental to any antiseptic evaluation.

There are recognized at least three possible mechanisms of antiseptic action. Several authors have suggested that antiseptics achieve their results by coagulation of protoplasm. Although this is probably a mechanism for heat sterilization and can be achieved by some antiseptics such as bichloride of mercury or phenol, effects on bacteria are produced at concentrations far below what would be required to produce coagulation. It appears probable that a very common mechanism is the poisoning by antiseptics of some essential enzyme substrate within the organism, and several such instances will be presented. At least three papers in this monograph report that antiseptics seem to cause bacteria to lose proteins and electrolytes to the surrounding medium, and these suggest a profound alteration in the permeability of the membrane surrounding the organism. Such effects in either direction (increase or decrease of permeability) would prevent the organism from maintaining equilibrium with its environment and would constitute a sufficient mechanism of death.

Finally, it is desired to emphasize the necessity of neutralizing any antiseptic before real knowledge can be gained of the location of that question mark in the figure. Any really useful dyestuff must be so strongly adsorbed on textile fibers that no amount of dilution or washing will remove it. It appears, similarly, that many effective antiseptics are so strongly held on organisms that the growth of untreated bacteria in the same medium after the completion of a negative test is wholly unreliable as an indication of death. Strongly adsorbed antiseptics must be neutralized by suitable chemicals or removed from bacteria by physical means before a finding of growth or no growth has any significance. Many will agree with several authors in this monograph that, for antiseptics to be used on and in tissues, the *in vivo* test is the final reference. For screening, and for antiseptics to be used on objects, surfaces, *etc.*, a definitive *in vitro* test is imperative, and we recently outlined the criteria to be applied.* A basic need is to have

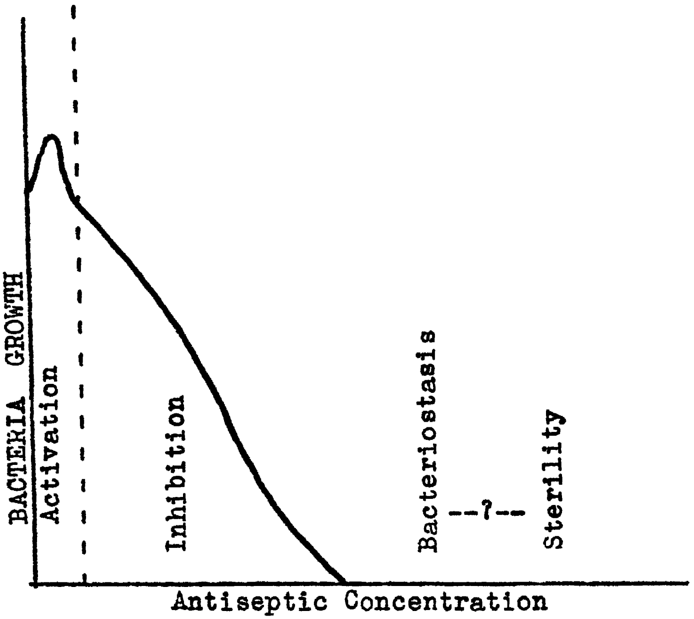

FIGURE 1.

TABLE 1
MECHANISMS OF NEUTRALIZATION

1. Insolubilization—mercury-sulphur compounds
2. Complex formation—copper-ammonia, phenol-iron
3. Desorption by better substrate—phenol—charcoal
4. Oxidation—reduction reactions—iodine—thiosulfate
5. Opposition of charge on colloidal particles and ions
6. Displacement from bacteria by strongly adsorbed innocuous substance

* DAVIS, H. L., A. BLOCH, & A. A. STONEHILL. Bull. Nat. Form. Comm. 16: 69-76, 1948.

for each antiseptic a chemical demonstrably able to neutralize its effect. It is true, however, that any bacteria-antiseptic complex, which finds in living tissues no chemical capable of neutralizing the adsorbed antiseptic, may still produce no infection, even though an effective neutralizer *in vitro* should show that the effect is only bacteriostatic.

In TABLE 1 is shown a partial list of mechanisms of neutralization. Many other examples of antagonism to and neutralization of antiseptics are included in the following papers. The earliest reference is that of Geppert, who in 1889 recognized the bacteriostatic action of such ions as mercury, silver, and copper; and the neutralization of mercuric ions by conversion to the insoluble sulfide has been demonstrated repeatedly.

The development of these antiseptic-neutralizer relationships will not be easy, but does appear essential and possible. No universal neutralizer appears likely, but there are coming into use neutralizers for classes of substances (thioglycollate). The cooperation of scientists from several fields will be required, and it is hoped that some of them will be colloid chemists. Let this monograph be a start in that direction.

THE METABOLIC ACTION OF STREPTOMYCIN

By W. W. Umbreit

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Our discussion is concerned with antibiotics. The discovery and exploitation of certain of these substances has been a bright spot in the work of the past decade. Since this was a period of desperate urgency, with wars and rumors of wars continually with us, one is likely to forget that the properties of these substances were close to unique. Indeed, if we did not know that substances such as streptomycin or penicillin existed, the properties ascribed to them, and which they do in fact possess, would be believed impossible. The useful antibiotics are new substances, unknown before our lifetime, and it is of some importance to learn all that we can about them. It is, therefore, appropriate to consider the biochemical aspects of the mode of action of some of these agents as part of the general knowledge of antibiotics, even though this information usually lags far behind practical knowledge of the usefulness of a given antibiotic and, of course, normally has very little influence upon its clinical use.

This paper is a somewhat restricted review of the site of action of streptomycin, particularly a summary of the conclusions reached by studies in our own laboratory. This is, very possibly, not a complete picture. It is, rather, a summary of the status at the moment. Much of the data is at present in the process of publication, and what we shall present here are typical illustrations of phenomena whose fuller description will be found elsewhere. Engaged in this work at one time or another have been Dr. Oginsky, Miss Smith, Dr. Solotorovsky, Mr. Tonhazy, and Mr. Waddell, and, collectively, we have reached the following views on the action of streptomycin.

At the start of this work, it was quite evident from the literature that streptomycin produced effects upon the metabolism of resting cells of bacteria. We began with the work of Geiger,[1] who showed that, with a particular strain of *Escherichia coli*, there was a marked stimulation of serine oxidation by the previous oxidation of fumarate. When streptomycin was present during the fumarate oxidation, the stimulation of subsequent amino acid oxidation was prevented. In a paper already published,[2] we were able to trace this effect to inhibition of some portion of the terminal respiration system of this organism. A reasonable explanation for the results would be the postulate that streptomycin inhibited the oxalacetate-pyruvate condensation and reactions analogous to it.

More direct study of this reaction has tended to confirm this conclusion.[3] Illustrative data are given in FIGURE 1. A resting cell suspension of *E. coli* was held in distilled water for one week in the refrigerator. This treatment markedly decreases oxalacetate decarboxylase. Such suspensions will oxidize pyruvate to the acetate stage, but, as may be noted, they oxidize oxalacetate only slowly. A mixture of oxalacetate and pyruvate is oxidized rapidly. Without streptomycin, this oxidation tends to go to completion; with streptomycin, the reaction is inhibited.

One may illustrate the concept of the site of action of streptomycin on *E. coli* by way of the diagram in FIGURE 2. The hypothesis was that it acted upon reaction A. Proof of this hypothesis is, however, a different matter. The best proof would be the actual isolation of the enzyme carrying out this reaction and the demonstration that it was inhibited by microgram quantities of streptomycin. So far, however, this has not been possible, and the enzyme appears to be quite refractory to the usual methods of isolation from bacterial cells. It was necessary, therefore, to rely upon other criteria. These consist of examining different types of systems in which the oxalacetate-pyruvate reaction would be evident in a different manner and the demonstration that streptomycin produces effects, in all of

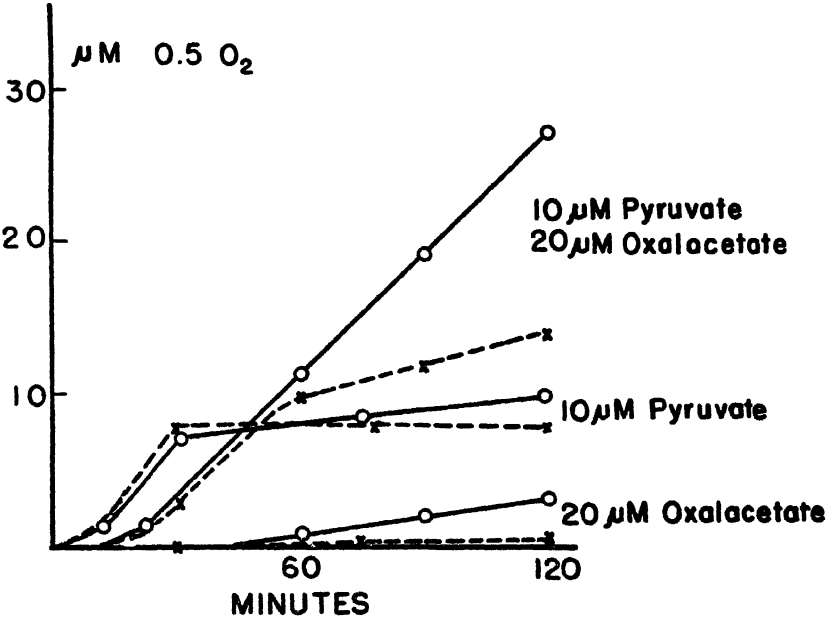


FIGURE 1. Oxidation of oxalacetate and pyruvate by aged suspensions of *E. coli*. Dashed lines represent observations in the presence of 20 µg. streptomycin per ml. (From Reference 3.)

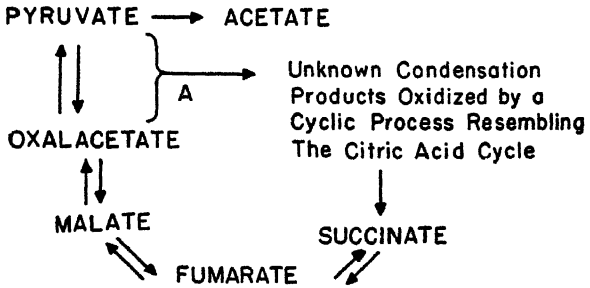
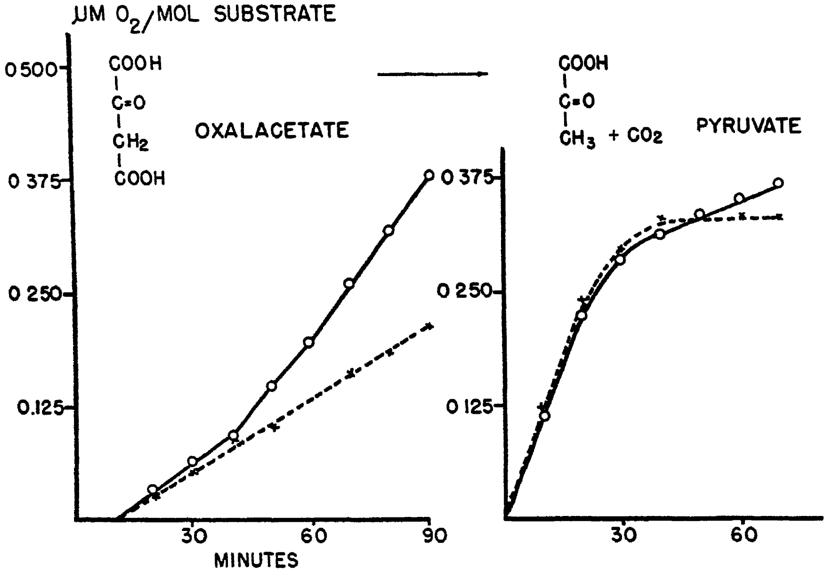


FIGURE 2. Diagram of the terminal oxidation process in *E. coli*. (From Reference 3.)

the systems, explainable by its inhibition of the oxalacetate-pyruvate reaction. It is probable that, eventually, alternative explanations for the phenomena can be thus eliminated, since possible alternatives for one system are not possible alternatives for another. We shall mention here five such systems.

First, the stimulation of amino acid oxidation by previous fumarate oxidation described by Geiger was shown[7] to be due to the formation of an intermediate, probably oxalacetate, which reacted with the keto acid derived from the amino acid. The conversion of serine to pyruvate and threonine to α-ketobutyrate and the condensation of these materials with oxalacetate seemed to be the most direct explanation of the phenomenon observed. The reaction was inhibited by streptomycin.



OXIDATION OF OXALACETATE AND PYRUVATE BY LIVING CELLS

FIGURE 3. Oxidation of oxalacetate and pyruvate by fresh suspensions of *E. coli*. Dashed lines represent observations in the presence of 20 µg. streptomycin per ml. (From Reference 3.)

Second, preparations made by vacuum-drying bacterial cells could occasionally be obtained which would hardly oxidize pyruvate.[2] Upon the addition (to the pyruvate) of fumarate or oxalacetate, rapid oxidation occurred, which was completely inhibited by streptomycin.

Third, freshly prepared cells of *E. coli* contain an active oxalacetate decarboxylase and rapidly convert added oxalacetate to pyruvate. Yet, as shown in FIGURE 3, there is a marked difference in the effects of streptomycin upon the two substrates.

Fourth, on aging such suspensions, the ability to convert oxalacetate to pyruvate is gradually lost. Such older suspensions, as shown in FIGURE 1, do not oxidize oxalacetate, but will oxidize a mixture of oxalacetate and pyruvate. This latter reaction is inhibited by streptomycin.

Fifth, it is evident that, if the pyruvate-oxalacetate reaction leads to the terminal respiration system, resulting in complete oxidation of the substrate, and if streptomycin prevents this reaction, then substances such as succinate, malate, *etc.*, would not be completely oxidized in its presence. In fact, since pyruvate is oxidized to acetate, one would expect these substances to be oxidized to acetate in the presence of streptomycin and much beyond this stage in its absence. This proves to be the case, as illustrated in TABLE 1.

These criteria taken together seem to us to constitute adequate evidence that streptomycin acts upon the oxalacetate-pyruvate reaction. We are as yet, however, quite unable to specify the exact details of this reaction; thus, we cannot specify at just what point of an admittedly complex series of reaction steps the streptomycin inhibits. Further, it seems quite impossible, as yet, to determine how the streptomycin exerts its effect. Beyond the facts cited, one cannot go, but it seems evident that it is on this complex of reactions, whatever they may be, that streptomycin exerts its effect. Yet, this information is sufficient to examine certain other problems.

TABLE 1
AMOUNTS OF OXYGEN UTILIZED ON VARIOUS SUBSTRATES

Substrate	*Moles O_2/mole substrate*		*Theory to acetate*
	Normal	*+ Streptomycin*	
Pyruvate	0.44	0.42	0.5
Oxalacetate	>0.87	0.5	0.5
Malate	>2.3	0.77	1.0
Fumarate	1.42	1.0	1.0
Pyruvate + Oxalacetate	>0.83	0.56	0.5

From Reference 3

Before discussing these, however, two items should be considered. In the first place, is the reaction inhibited the right one? Here, three criteria may be applied. First, the reaction is completely inhibited by concentrations of streptomycin necessary to obtain complete inhibition of the growing cell, in our strains, of the order of 10 to 20γ per ml. Second, only those streptomycin derivatives, streptomycin, dihydrostreptomycin, and mannosido streptomycin, which inhibit growth, inhibit the reaction. Third, a wide variety of other reactions in the cell are not in the least affected by even very high concentrations of streptomycin. The second item, which is certain to arise, is whether streptomycin influences "active acetate" and whether the results reported are not referable to streptomycin inhibition of either "active acetate" formation or activity or the oxidation of the substance formed by its condensation with oxalacetate. The point is certain to be debated for some time to come, and this is not the time to do it. All attempts, however, to implicate "active acetate" have so far failed. By the term "oxalacetate-pyruvate reaction," we imply a complex of reactions, the condensation of oxalacetate and pyruvate and the oxidation of the unknown intermediates formed, but we do not exclude the formation of a transitory 2-carbon intermediate. We feel that, if it does exist, its origin is directly from pyruvate and that it does not exchange with acetate to any great extent. The problems of the exact nature of the reaction and the exact way in which streptomycin inhibits it remain for the future.

The results, however, do permit one to examine some other problems. For example, what happens in the resistant strains?[1] Data on the oxalacetate-pyruvate reaction are given in FIGURE 4. It is evident that, in so far as one can measure these reactions, the resistant strain does not possess it. Strains dependent on streptomycin likewise show no evidence of the presence of an oxalacetate-pyruvate reaction. Thus, it appears that strains capable of growing in the presence of streptomycin have eliminated the reaction inhibited by the drug. Having dispensed with this process, it is presumed that they have developed some alternative, but just what this may consist of is at present quite obscure.

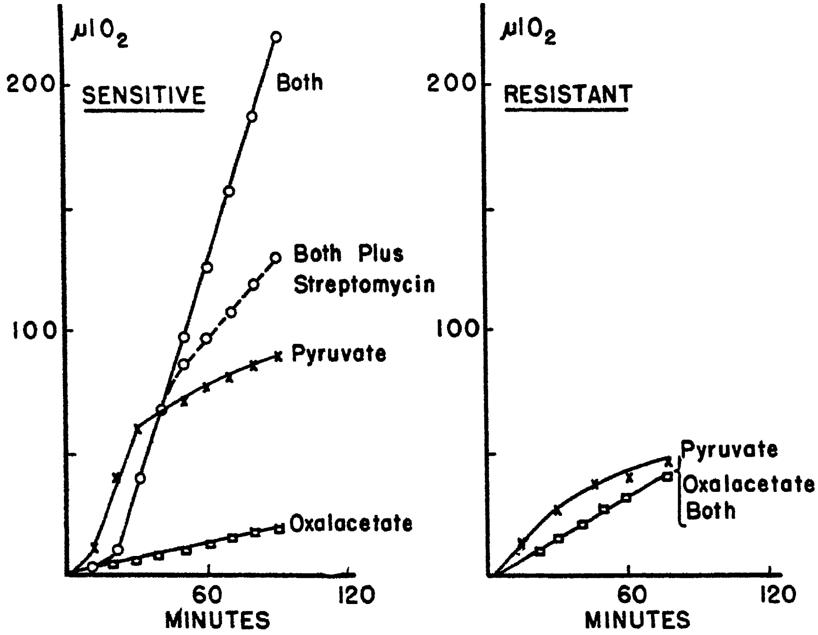


FIGURE 4 Comparison of reactions in sensitive and resistant strains With the resistant strain, identical results are obtained in the presence or absence of streptomycin (From Reference 4.)

Quite possibly, however, the most important problem in the action of antibiotics is their ability to enter the animal body and inhibit or kill the susceptible bacteria therein without harm to the host, or, at most, anything approaching comparable harm. It is obvious the oxalacetate-pyruvate reaction is an important one in the animal. Why does not streptomycin inhibit it as well?

The enzymes carrying out this reaction in the animal cell are localized largely in the mitochondria. Within the past few years, it has been possible to prepare cell-free mitochondria in an enzymatically active state. When this is done and streptomycin is supplied, no inhibition of the oxalacetate-pyruvate reaction is evident, except at exceedingly high levels of streptomycin. When, however, streptomycin is allowed to penetrate into the mitochondria,[5] the oxalacetate-pyruvate reaction is inhibited by relatively low levels of streptomycin, partial inhibitions being achieved by concentrations of the order of 10–20γ per ml., complete inhibition by 100γ per ml. There is the additional observation that streptomycin in the body seems to be largely in the extracellular fluids. Little, if any, penetrates most cells. One may, therefore, suppose with some assurance that streptomycin inhibits the oxalacetate-pyruvate reaction in the animal as well as in the bacterial cell but that, in the animal, it is prevented from reaching the sensitive enzyme by barriers, presumably permeability barriers, at the cell wall and at the mitochondria.

Streptomycin's action against the tubercle organism is perhaps a special case.[6] In these bacteria, it has so far been impossible to demonstrate an oxalacetate-pyruvate reaction. Streptomycin does, however, inhibit the oxidation of the higher fatty acids, from C_{12} on. It is, of course, evident that not much is known about the metabolic pathways in acid-fast organisms, but, in so far as we have been able to trace the oxidative pathways of the higher fatty acids in this organism, it appears that a mechanism analagous to the oxalacetate-pyruvate reaction is inhibited. Perhaps fatty-acid metabolism has some special significance for the growth of the acid-fast organisms, and perhaps this is the reason for the special action of streptomycin in this group.

The data and discussion presented make it evident that streptomycin acts upon a specific type of reaction. We do not know the mechanism of this reaction, except that it is undoubtedly a complex of several reactions and that it is apparently a quite important one, not only in the bacteria, but in the animal as well. It is, of course, uncertain whether this is the so-called mode of action of streptomycin. All that one can conclude with surety is that it is a reaction inhibited by streptomycin. At the moment, there appear to be at least two other possible sites of action of streptomycin. One is the reaction with certain desoxyribose nuclei acids.[7] The other is the inhibition of diamine oxidase recently reported by Zeller *et al.*[8] and obviously there may be still others unknown to us. The problem of which one does the real damage to the organism is, of course, not solved, but at this stage of our knowledge the question itself has very little meaning. What is important is that the antibiotic does act, in a rather specific manner, against certain metabolic reactions. These, even though we do not know their details, can be measured, and, in this sense, we have made some progress from the somewhat vague inhibition of growth to knowledge of inhibition of particular sites of metabolic activity.

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THE EFFECT OF PENICILLIN UPON PROTEIN SYNTHESIS BY BACTERIA

By Rollin D. Hotchkiss
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The emergence of such an important chemotherapeutic agent as penicillin naturally poses the question of the mode of its action. There are at least three directions from which this question comes. First, those people who are concerned with the practical use of a therapeutic aid inevitably wish to understand the basis for its activity in order to know better how to apply the agent—in what amounts, at what time intervals, at which sites, and so on. Secondly, those who are interested in the development of new chemotherapeutic agents wish to learn how a known potent agent acts, in order to surmise the nature of its "active group" and the possibility of synthesizing useful modifications, or to devise screening tests to judge the value of new preparations. A third group, concerned with cell physiology, will be interested in the evidence of vulnerable points in cell biochemistry at which foreign agents can be utilized to control growth or metabolic processes. Having looked particularly along this last direction in our laboratory, we have come to certain observations of the effect of penicillin upon bacterial nitrogen metabolism. The best reason for placing these findings in this monograph is the hope that someone may be helped by them to make an as yet unanticipated advance along the first two directions mentioned: the better use of known penicillins or the development of new substances with a related action.

The information already available concerning the mode of action of penicillin can unfortunately only be briefly summarized here. The fundamental findings of Hobby, Meyer, and Chaffee,[1] Lee, Foley, and Epstein,[2] Hirsch,[3] Chain and Duthie,[4] and others, have indicated that it acts upon susceptible bacteria when they are growing, but not when they are in a resting phase. In an environment suitable for proliferation, bacteria exposed to penicillin rapidly lose viability, as demonstrated in a growth test by subsequent transfer to a suitable environment not containing penicillin. The rate of loss of viability (which may be considered the rate of killing) is dependent upon the penicillin concentration up to an optimal level, usually in the neighborhood of one or two international units per cubic centimeter. This is in contrast to the impression gained from the common all-or-none test, in which the failure to reach a visible turbidity or colony size in 24 hours is considered to be complete inhibition. The latter test disregards the large amount of invisible, or even visible, proliferation which may have occurred and been superseded by loss of viability or lysis. Nevertheless, it has given rise to the prevalent notion that the lethal action of penicillin is maximal at a few hundredths of a unit per cubic centimeter.

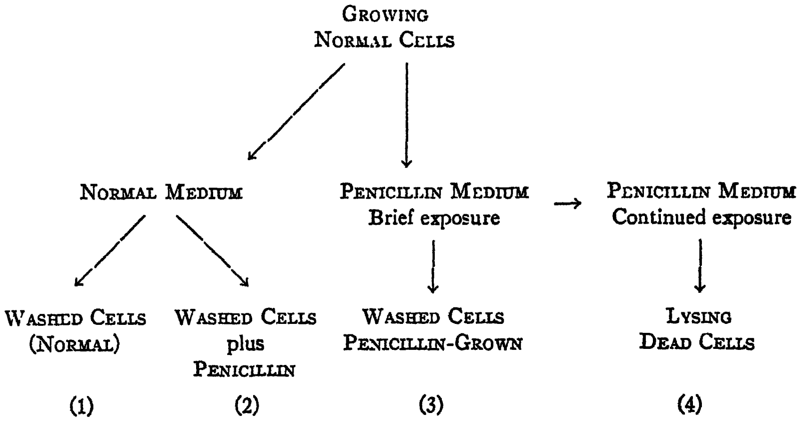
In some of the investigations mentioned,[3,4] and subsequent ones,[5,6] respiration or other metabolic activity was shown to be a more or less suitable measure of the growth or failure of growth in a bacterial culture. In all investigations through the last seven years, however, there has been virtually no clear-cut observation that penicillin could affect a metabolic system in anything but a growing cell. In one exception, Krampitz and Werkman[7] had observed that the slowly developing breakdown of ribonucleic acid by starved washed bacteria was prevented by penicillin. Otherwise, washed bacteria, lyophilized bacteria, bacterial enzyme preparations, *etc.* are not known to be affected by penicillin.

Nevertheless, new light was recently thrown upon the mode of action of penicillin, largely through the efforts of one group of workers. In 1946 and the following year, Gale and Taylor[8, 9] reported that staphylococci grown in media containing penicillin and prepared as washed cell suspensions were rendered incapable of concentrating glutamic acid from the external environment. Only in this metabolic activity, of several investigated, were penicillin-grown cells different from normal cells. The interference with the ability to accumulate this amino acid could be observed also in the cells during culture in penicillin-containing medium, but, when penicillin was not added until after the bacteria were removed from growth medium and prepared as washed cells, it had no effect upon their glutamic acid concentrating function. Obviously then, this system in itself was not vulnerable to penicillin, and some primary injury occasioned by penicillin in growing cells must have led to the interference with this concentrating system. Later work from Gale's laboratory[10, 11] indicated a correspondence between penicillin sensitivity and dependence upon preformed amino acids in general.

From a biochemical point of view, the observation of any effect of penicillin upon growing cells is complicated by the simultaneous and subsequent occurrence of numerous other reactions: any or all of the reactions of normal growth, secondary processes resulting from the distortion of normal metabolism, *etc.* Thus, it would be difficult indeed to discover the site of primary injury. We are approximately in the situation of an investigator, who knows little of the mechanics involved, trying to examine a crashed airplane for evidences of the initial mechanical failure which led to the accident. Since the mechanical system is now grossly disrupted and he does not understand it very well anyway, he naturally places greatest weight upon any testimony obtainable as to events before the crash, the earlier, the better. In studying mechanisms of antibacterial action, we are led to do likewise, that is, look for injury occurring in an enzymatic preparation or washed cell system before secondary processes have had an opportunity to confuse the observer. The general types of cell preparations available are indicated in TABLE 1. This scheme suggests the relatively greater significance of findings made with washed cells prepared from normal media. The difficulty has been that penicillin has almost universally failed to affect such cells.

At first sight, it might appear that penicillin should not be expected to affect the metabolism of washed cells, since it has been reported not to reduce the viability of resting bacteria. We must remember that this means merely that an irreversible process does not occur and, furthermore, that only a few kinds of resting bacterial preparations were tested (cells in saline, in diluted media, cells at low temperature, *etc.*). It is still almost beyond our power to comprehend, or to investigate in any detail, the composite of biochemical reactions that constitute growth. Nevertheless, it is certainly reasonable to assume that, if some part of the process of growth is vulnerable to penicillin, this particular partial growth process can eventually be studied in the proper kind of resting cell or enzyme preparation, without the occurrence of growth itself.

TABLE 1


GROWING NORMAL CELLS
NORMAL MEDIUM
PENICILLIN MEDIUM Brief exposure
PENICILLIN MEDIUM Continued exposure
WASHED CELLS (NORMAL)
WASHED CELLS plus PENICILLIN
WASHED CELLS PENICILLIN-GROWN
LYSING DEAD CELLS
(1)
(2)
(3)
(4)

GENERAL RESULTS OF COMPARISON OF ABOVE PREPARATIONS:

Comparing (1) with (4) reveals differences between living and dead cells.
Comparing (1) with (3) reveals differences between living and dying cells.
Comparing (1) with (2) reveals differences occasioned by penicillin.

The discovery that respiring washed staphylococci were able to take up amino acids with the formation of cellular protein[12] made available a partial growth process and furnished an opportunity to study the effect of penicillin upon protein synthesis in a nonproliferating system. When first tested in this way in 1946, penicillin appeared to have no influence on protein synthesis. The appearance of the reports of Gale and Taylor, already mentioned, to the effect that cells grown in penicillin had lost the ability to concentrate free glutamic acid, stimulated us to further experimentation in this direction.

In staphylococci grown, like those of Gale and Taylor, for a few hours in low concentrations of penicillin, we have studied the utilization of glutamic acid, other amino acids, and simple mixtures of these.[13] Inhibition of glutamic acid uptake was not more than 15 per cent, and, therefore, far different from the virtually complete inhibition observed by the original authors. Since the respiratory rate in penicillin-grown cells was falling faster than that of the normal cells, and amino acid uptake is dependent upon respiration,[12] it is possible that the small inhibition of glutamic acid utilization was a secondary result. The present conditions differed from those of the original experiments in that a different strain of staphylococcus was used, it was grown in media not deficient in amino acids, and crystalline, rather than amorphous, penicillin was employed. Taken together, these two somewhat conflicting results seemed to indicate that the primary injurious action of penicillin is not upon glutamic acid utilization, but it might, under some conditions, lead to the inhibition of this function.

At the same time, it was also possible to test the utilization of other mixtures of amino acids. In none of the mixtures investigated did penicillin-grown cells use up appreciably less amino nitrogen than normal cells. By and large, therefore, the rate of utilization of various amino acids by staphylococci was not affected by penicillin added to washed bacteria, nor was it necessarily altered in cells grown in the presence of the agent.

A quite different picture was obtained when, instead of the disappearance of amino nitrogen from the environment, the total nitrogen distribution was followed.[13, 14] As already mentioned, normally the respiring washed cells will take up amino acids with production of cellular protein, the latter increasing as much as 30 per cent above its initial value. In the presence of penicillin, however, the washed cells do not increase in nitrogen content, although, as already described, they cause the free amino nitrogen to disappear just as fast as do the untreated cells. This can only mean that nitrogenous substances not containing amino nitrogen are accumulating in the extracellular solution when penicillin is present. Analysis of the supernates confirms this expectation, the amount of non-amino substance being approximately equivalent to the amount of amino nitrogen which, in the absence of penicillin, the normal washed cells will have converted into cellular protein.

The production of this nonamino material is clearly attributable to biologically active penicillin. Although 10 units or less of crystalline benzylpenicillin per milligram of bacteria will bring about the formation of this material, five times this quantity of inactivated penicillin did not have any such effect. This is true of penicillin inactivated by acid, akali, or heating in neutral solution, procedures which are known to produce, in general, three different types of degradation. The active antibiotic is equally effective in producing the nonamino substance at the lowest and the highest levels so far tested. Since the bacterial suspensions used, however, are usually several thousand times as concentrated as those used in a bacterial growth inhibition test, the lowest concentration of penicillin tested up to this time has been 30 units per cubic centimeter. With respect to bacterial mass, this amount is less than 1 per cent of that maximally inhibiting growth, while, on a volume concentration basis, it is fifteen to twenty times larger.

The nonamino, nitrogenous material produced in the presence of penicillin can be concentrated by precipitation and adsorption techniques. In concentrates, it is found to give strongly the biuret reaction characteristic of peptides and proteins. The solubility and precipitability suggest that it is largely in the form of higher peptides. Since the biuret reaction is a measure of peptide bonds, the amount of the unidentified peptide can be estimated. Such estimates indicate that the amount of peptide nitrogen formed rather closely corresponds to the amount of amino nitrogen disappearing from the amino acids. Thus, in two separate experiments, in which 2.88 and 5.15 milligrams of amino nitrogen were utilized, the amounts of peptide nitrogen recovered were 2.9 and 5.0 milligrams, respectively. Cells not exposed to penicillin produced in the same experiments only 20 per cent and 13 per cent as much extracellular peptide.

The findings just summarized suggest the hypothesis that penicillin interferes with the bacterial synthesis of protein from amino acids, and the process is blocked at such a point that peptide intermediates accumulate instead. However, it still remains possible that the peptides found are abnormal intermediates, as, for example, peptides containing penicillin, or that they are degradation products of a damaged cell. It can only be pointed out that the amount of peptide formed is remarkably close to the amount of amino nitrogen utilized, and that it is not formed unless amino acids are supplied to the washed bacteria. Further conclusions can be drawn when we are able to describe more fully the chemical and biological properties of these interesting products formed when washed bacteria utilize amino acids in the presence of penicillin.

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THE COLLOIDAL NATURE OF ANTIBIOTICS

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Just about thirty years ago, it was discovered that salts of higher fatty acids will react like simple electrolytes if present in great dilutions, but in higher concentrations will exhibit properties typical of the colloidal state of matter.[12, 13] It was at that time that the term "colloidal electrolyte" was introduced into scientific terminology. Colloidal electrolytes are salts in which one of the ions has been replaced by a highly charged, heavily hydrated ionic micelle, which exhibits equivalent conductivity that is not only comparable with that of a true ion but may even amount to several times that of the simple ions from which it has been derived. In other words, this ionic micelle is a typical, but very highly charged, colloidal particle of very great conductivity. Some of the very numerous substances which must be recognized as belonging to this group are the protein and gelatin salts, the higher sulphonic acids, in fact, most substances of high molecular weight or those containing long carbon chains which are capable of splitting off an ordinary ion.[5] Just as very little credence was given to these statements at that time, a discovery reported quite recently, that most antibiotics must also be classified as colloidal electrolytes,[6, 8] has caused considerable controversy among scientists.[9, 15]

In a paper on "Colloid Phenomena In Bacteriology," written twenty-seven years ago,[18] one finds the following: "However diverse the action of growth stimulants or however complicated the mechanism by which death is caused in the microorganisms, nevertheless, in many cases, at least in the preliminary stages, the bacteria may be regarded as simple colloidal systems. The more recent developments in the study of colloidal systems have emphasized the important influence of the interfacial surface on colloidal reactions. With bacteria presenting the extended surface of colloidal systems, adsorption at the interface is of primary importance. If any substance added to the medium lowers the interfacial surface tension, then the surface concentration, in accordance with Gibbs's equation, will be greater than the bulk concentration. It is evident that if the added solute has a great effect on the surface tension, the surface concentration may become quite high. The inhibiting materials produced during bacterial growth may be soaps, fatty acids, and higher aliphatic alcohols. The adsorption of organic substances from solutions by bacteria is to be ascribed to the presence of certain groups in the molecules. The growth rate of microorganisms is greatly influenced by the concentration at the bacterium's surface of the various substances present. The surface concentrations may already be considerable, even when scarcely detectable amounts are present in the bulk of the medium."

Taking this citation into consideration and reviewing it critically in the light of what has been said about colloidal electrolytes, it seems obvious that it did not receive the attention it deserved. If it had, more emphasis would have been placed on the part the colloidal properties of antibiotics play in regard to their activity.

Chemical and Physical Factors

Of all chemical formulae which have so far been proposed for penicillin, the one represented schematically in FIGURE 1 seems the most acceptable.[17] The different R chains in the preparations now available are given in TABLE 1. Besides these differences, the hydrogen ion of the hydroxyl group in the acid radical can be exchanged for cations like sodium or calcium, resulting in the formation of salts. Molecular weights of these preparations have been determined by measuring the freezing point depression and the diffusion constant.[10] [20] The molecular weights so obtained vary between 312 and 490. Conductivity measurements of aqueous solutions of sodium benzyl penicillate—penicillin G—of extremely low concentration have also been carried out.[8]

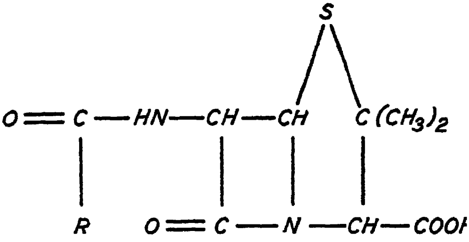

FIGURE 1. Basic formula for salts of penicillic acids.

TABLE 1

Penicillin	*R*
F	$CH_3 \cdot CH \cdot {}_2CH{=}CH{-}CH_2{-}$
G	$C_6H_5 \cdot CH_2{-}$
X	$p{-}HOC_6H_4 \cdot CH_2{-}$
Dihydro-F	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2{-}$
K	$CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2{-}$

On the basis of all these data, it has been concluded that penicillin salts in solution behave as completely dissociated electrolytes.[11] In dealing with the sodium salt, the results were found to be in line with those to be expected from a 1:1 valence type electrolyte, although deviations due to ion size and interactions had to be considered as possible.[20]

Medical Factors

It has been reported that the electrostatic charge of bacteria undergoes a change upon the addition of antibiotics and that the antibiotic activity of sodium penicillin can be materially increased upon the addition of minute quantities of cobalt chloride.[16]

A few years ago, some work was carried out to ascertain whether electrokinetic potential studies of bacteria to which bacteriostatic agents have been added could shed some light on the question of what causes their activity.[3] Although a pronounced change in the potential of the bacteria was noticed upon addition of the agent, the conclusion was drawn that this could not have been the result of adsorption of the bacteriostatic agent on the surface of the bacterium. This opinion was based on the observation that the electrokinetic potential of quartz particles added to a similar medium was not affected.

More recently, medical circles reported that penicillin causes a change of the electrostatic charge of susceptible bacteria, which is measurable in terms of their zeta potential. It has also been claimed that bacterial growth in penicillin assay plates showed enhancement in electronegative and inhibition in electropositive zones.[3] No conclusions, however, as to the cause for these electrokinetic phenomena have as yet been drawn from these results.

Colloid Chemical Aspects

If these statements are reviewed from a colloid chemical point of view, it is clear that they cannot be considered as convincing evidence for proving that penicillin acts like a true electrolyte when put into solution. Therefore, a review of some colloidal aspects of penicillin might be appropriate at this time.

Even very dilute solutions of purified sodium penicillin G reveal a Faraday-Tyndall cone when observed in the ultramicroscope.[8] This cone becomes the more pronounced the higher the concentration of the solution, or when the temperature is decreased. If 0.2 mg. $CoCl_3$ is added to 1 cc. of a crystalline sodium penicillin G sol of 15,000 units, the number of colloidal micelles observable in the ultramicroscope is considerably increased (FIGURE 2). The same is the case if aluminum or thorium salts are added to the penicillin sol. When subjected to ultracentrifugation, the system separates into two distinct layers. In the ultramicroscope, one shows a high concentration of clearly discernible anisometric particles, whereas the other exhibits, at the very best, a faint bluish light cone. Microanalysis of the two fractions reveals that the highly colloidal fraction contains most of the added polyvalent cations, whereas the optically clear layer contains sodium and chlorine ions in solution. Quantitative analysis of both fractions has shown that the amount of sodium ions found in the dispersion medium is exactly of the 3:1 ratio for the amount of cobalt or aluminum, and of the 4:1 ratio for the thorium ions which have been added. An ion exchange had taken place which, due to the less pronounced hydration of the multivalent cations, resulted in a greater number of formed penicillin micelles.

Exactly the same observations have also been recorded when studying the calcium chloride complex of streptomycin.[9]

Electrophoretic studies[2] and zeta potential determinations of sodium and cobalt penicillin sols revealed that the colloidal particles or micelles migrate to the anode of the electric field and therefore are negatively charged. In contradistinction thereto, the ultramicrons of aluminum and thorium penicillin migrated to the cathode. This is a clear indication that a charge reversal of the colloidal micelle had taken place. The addition of stronger electrolytes, as, for example, acetic acid or a 30 per cent solution of ammonium nitrate, will result in pronounced, but nevertheless, reversible agglomeration of the penicillin micelles (FIGURE 3). Electrophoretic studies of streptomycin sols reveal that these particles also carry a net negative charge.[2, 9]

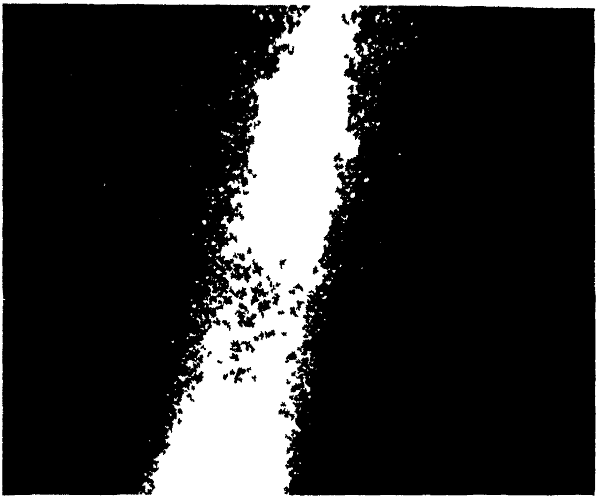

FIGURE 2 Sodium Penicillin G (15,000 units) + 0 2 mg $CoCl_2$ (slit ultramicroscope).

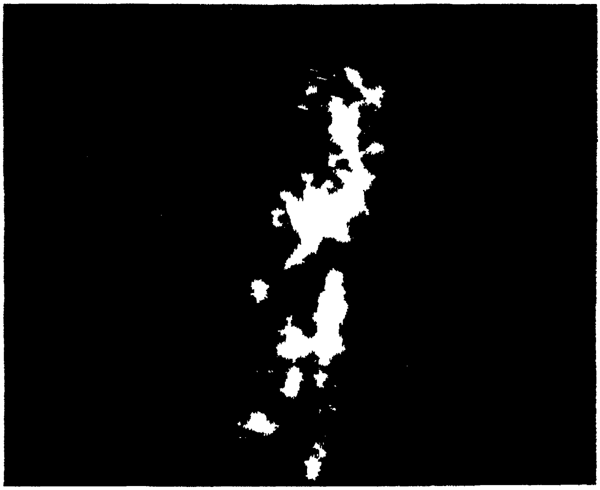

FIGURE 3 Sodium Penicillin G (10,000 units) and ammonium nitrate

A considerable amount of work has been devoted to determining if and to what extent the addition of penicillin salts and other antibiotics will affect the surface tension of water.[7] The pendent drop method was used for the present investigation, since there can no longer be any doubt that it is not only the most accurate but also the most ideally suited method for this type of work. Experiments carried out so far included sodium and potassium salts of penicillin G and sodium salt of penicillin dihydro F. The concentrations studied so far ranged from 2,000 to 60,000 units/cc. The temperatures at which the determinations were made ranged between 13° and 37° C. The surface tension was not only determined immediately after drop formation but also after the drop had been allowed to age, up to fifteen minutes. The experimental conditions employed insured constant humidity, essentially no mass transfer between the drop and its surroundings, and good temperature control (±0.2° C.). Of all preparations investigated, sodium penicillin G reached the lowest values of surface tension in the shortest period of time. It therefore must be classified as the most surface-active of all the penicillin salts investigated. The potassium salt was the least surface-active. The surface tension of both preparations was found to drop rapidly during the first 100 seconds and then to decrease more gradually. This checks with the statement made, a few years ago, by bacteriologists trained in colloid chemistry,[19] that capillary-active cations exert antibiotic properties towards negatively charged bacteria.

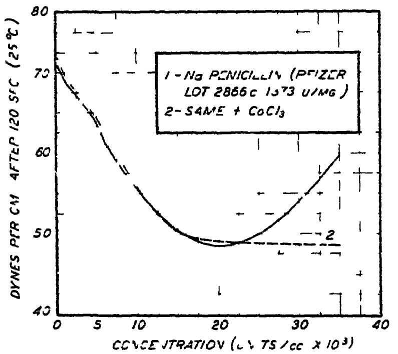

FIG 4

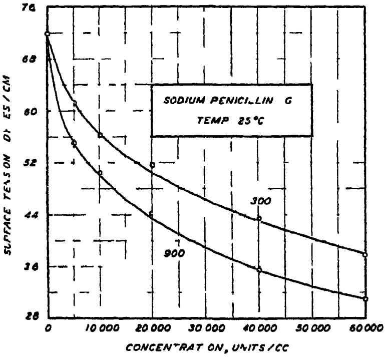

FIG 5

FIGURE 4 Surface tension *vs* concentration of sodium penicillin G with and without addition of cobalt chloride [6]

FIGURE 5 Surface tension of aqueous sodium penicillin G at various concentrations and times [9]

There is, however, one discrepancy between previously reported data and results obtained more recently which deserves attention. Surface tension concentration curves of soap solutions passed through a minimum at low concentrations.[14] The same observation has been reported for penicillin solutions.[6] Some investigators pointed out that the occurrence of such minima is probably due to the presence of impurities.[15] The latest results, based on penicillin preparations of a more recent date, did not exhibit the minimum in the surface tension *vs.* concentration curves. These recent data, however, show a far more pronounced decrease in surface tension *vs.* concentration than has been reported so far. This is clearly evidenced by comparing FIGURE 4[6] with FIGURES 5, 6, and 7.[9]

Surface tension determinations of a streptomycin sol gave no indication of any pronounced surface activity. This is most probably due to the covalent calcium ion and its low degree of hydration and the molecular structure of this compound,[20] which in itself offers little reason, if any, why this compound should reveal any marked capillary activity. It has been found, however, that the antitubercular activity of streptomycin could be increased over a thousandfold if its molecule was coupled with a capillary-active substance.[4] Viscosity determinations with aqueous solutions of varying concentrations exclude any argument that we are dealing with a macromolecular structure, because the latter would show a very pronounced and rapid increase in viscosity with concentration. This is not the case, however, as shown in FIGURE 8.[9]

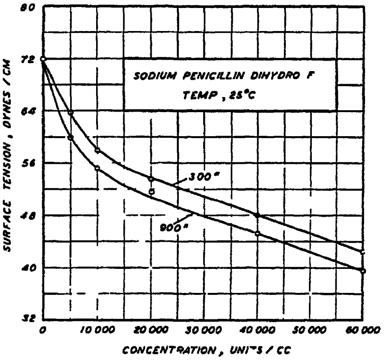

FIG. 6

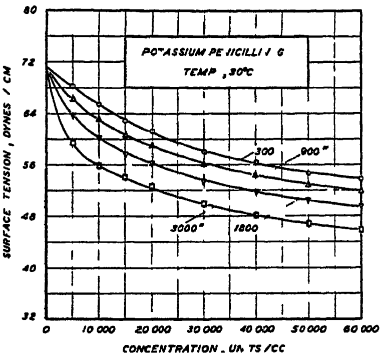

FIG. 7

FIGURE 6 Surface tension of aqueous sodium penicillin dihydro F at various concentrations and times [9]
FIGURE 7. Surface tension of aqueous potassium penicillin G at various concentrations and times [9]

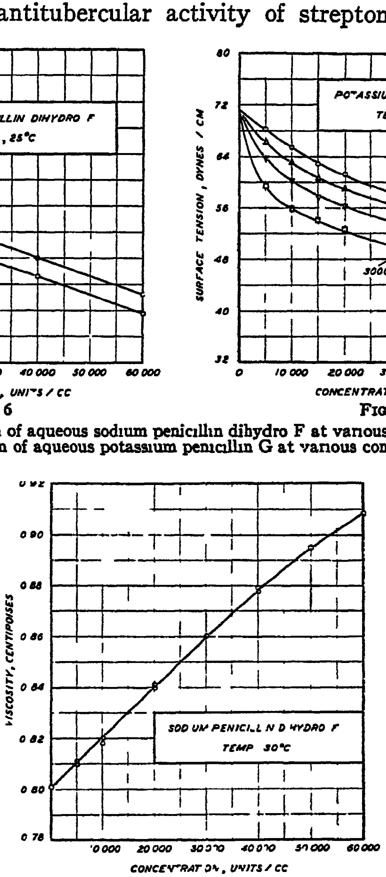

FIGURE 8 Absolute viscosity of aqueous sodium penicillin dihydro F at various concentrations.[9]

The specific conductance, expressed reciprocally in ohms per centimeter, increases linearly with concentration (Figure 9).[9] However, the equivalent conductance, expressed in ohms^{-1} per centimeter^2 (FIGURE 10),[9] reveals even more clearly that we are not dealing with a strong electrolyte, since its plot against the square root of the concentration revealed a pronounced shift at a concentration corresponding approximately to 1,000 units/cc. From a colloid chemical point of view, this clearly indicates that the equivalent conductance below this concentration, if it is corrected for the conductivity due to the water, corresponds to that of a solution composed of individual molecules which are dissociated into positive and negative ions.

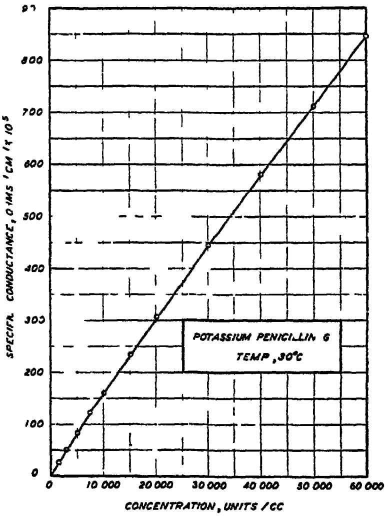

Figure 9. Specific conductance of aqueous potassium penicillin G at various concentrations.[9]

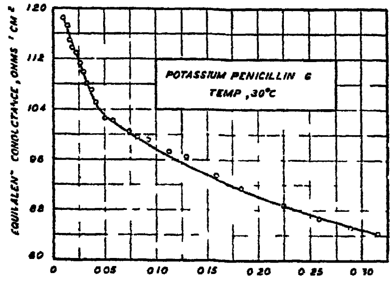

Figure 10. Equivalent conductance of aqueous potassium penicillin G at various concentrations[9]

The concentration at which the curve shifts may therefore be considered as the threshold of micelle formation. It is this concentration which has just revealed the presence of colloidal particles in the slit ultramicroscope.

Ultramicroscopy and Ultraviolet Microscopy

When a drop of a liquid culture medium containing *Staphylococcus aureus* is placed under the ultramicroscope, the spherical bacteria are seen to be moving in rapid Brownian motion. If a drop of a solution of sodium penicillin G of a concentration just beyond the threshold reported above is added to this solution, it is possible to observe visually how the penicillin micelles interact with the bacteria. Very soon, either each individual bacterial cell becomes coated with one or more visible penicillin micelles or the bacteria agglomerate and more penicillin micelles are then adsorbed on the surface of the resulting cluster. Brownian motion comes to a stop and the cells appear quite lifeless.

Similar results can be obtained if a sample from a streak culture on agar is drawn off with a wire loop, smeared on the slide, diluted with water, and the penicillin solution then added. As the penicillin diffuses through the preparation, clustering becomes immediately noticeable (FIGURE 11).

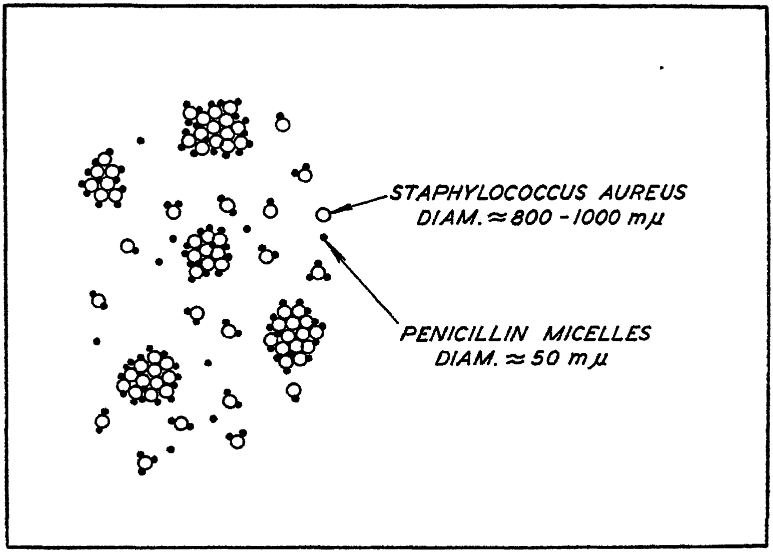

FIGURE 11. Interaction between *Staphylococcus aureus* and aqueous sodium penicillin G.[9]

The same tests were also carried out with cultures of *Staphylococcus aureus* and penicillin under an ultraviolet microscope. *Staphylococcus aureus* does not fluoresce under ultraviolet illumination, but benzyl penicillin does so very pronouncedly. Its color of fluorescence is blue. After a very short time, the fluorescence became more and more pronounced on the surface of the individual bacterial cells[6, 8] and later at the surface of the cell clusters and on points of contact between the cells within a cluster. In proportion to the increase in fluorescence at these interfaces, the fluorescence of the continuous phase of the system decreased, indicating that the dispersed phase of the penicillin sol had been primarily concentrated at the interfaces. This is certainly the best experimental evidence for a theory of the action of antiseptics which was written two years ago:[1] "It is emphasized that the first requirement of an antiseptic appears to be that it shall be adsorbed on the microorganism, and many demonstrations of maximum antiseptic effects depend on discovering conditions which comply with the colloidal requirements for achieving high adsorption. What happens after that to produce inhibition or death is not clear. Heavy metal salts, organic toxic agents, solvents, *etc.* may produce agglomeration or coagulation of protoplasm. With oxidizing agents, sulfa drugs, and antibiotics, the more probable mechanism appears to be the poisoning in the organism of some vital substrate needed for the catalysis of essential metabolic processes. Such adsorption may be of low magnitude and effect and easily reversed by dilution, or the antiseptic may be so tenaciously held as to make necessary its displacement from or neutralization on the organism to permit renewed growth."

Conclusions

Evidence based on ultramicroscopic observations, electrokinetic studies, cation exchange reactions, conductivity measurements, viscosity, and surface tension determinations has been offered to prove that antibiotics must be classified as colloids.

These results clearly indicate that the action of antibiotics like penicillin and streptomycin depend on their colloidal properties.

It therefore seems only logical to assume that the greatest activity could only be achieved if it could be ascertained how the strongest adsorption could be accomplished. It would also seem advisable to approach problems pertaining to the effect of antibiotic agents more from the colloid chemical point of view than has so far been the case. This might lead to the discovery of still more powerful antibiotics than are now available, or even of synthetic compounds of specific antibiotic activity, starting with capillary-active colloidal electrolytes.

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NEWER ANTIBIOTICS: FACTORS INFLUENCING THEIR ANTIMICROBIAL ACTIVITY

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The mechanisms by which antimicrobial agents function has long been of interest to microbiologists. This interest, in part, has been due to the belief that an understanding of the factors responsible for the mechanisms of action of these substances may result in the development of better chemotherapeutic agents. To date, essentially all studies on their mode of action have concerned themselves with penicillin and streptomycin. Innumerable other antimicrobial agents have been described. Many more have been observed but not reported in the scientific literature. Few attempts have been made, however, to determine the mechanisms by which they function.

Sulfanilamide, although not an antibiotic in the true sense, was one of the first antimicrobial agents to receive widespread attention from the chemotherapeutic standpoint. Sulfanilamide and the sulfonamide group of compounds in general are bacteriostatic agents which exert a strong antibacterial action against certain of the Gram-positive group of organisms.[1] It is well recognized that the sulfonamides act by inhibiting growth rather than by killing, that their action is influenced by the number of organisms present, and that they are relatively ineffective against large numbers of cells. The sulfonamides, furthermore, exert their effect only after a lag period lasting from five to seven hours. Many of the sulfonamides have a structure essentially similar to para-aminobenzoic acid and are inhibited by the presence of this substance. Although it is believed by some investigators that the sulfonamides exert their effect by inhibiting bacterial respiration, others believe that they act by virtue of their similarity to the essential metabolite, para-aminobenzoic acid. The weight of evidence perhaps favors the latter theory.

Penicillin, in contrast to the sulfonamides, may exert either a bactericidal or bacteriostatic effect depending upon the experimental conditions.[2] Penicillin is an organic acid which is active in low concentrations against many microorganisms. Early studies indicated that, under the influence of high concentrations of penicillin, the number of organisms decreases at a constant rate until 99 per cent of the organisms have been destroyed. The rate of killing varies with different organisms and with the concentration of penicillin used. Its action is influenced only slightly by the number of organisms present and by environmental conditions. Its action is specifically inhibited, however, by penicillinase, an enzyme capable of destroying penicillin. Penicillin is effective only when active multiplication takes place. These observations have been confirmed abundantly in recent years,[3] and, in view of the fact that penicillin approaches the perfect chemotherapeutic agent more nearly than any other substance known, they may be used to advantage as a basis for comparison of other compounds.

Streptomycin, in contrast to penicillin, is an organic base which likewise is active in low concentrations against many of the Gram-negative microorganisms and against the acid-fast bacilli.[4] It is predominantly bacteriostatic in nature although bactericidal activity may be demonstrated under appropriate conditions. The activity of streptomycin is influenced by many more variables than the activity of penicillin, and it is recognized that, other environmental factors being constant, the activity of streptomycin varies markedly with the concentration of antimicrobial agent used, the size of inoculum, the medium, and pH. In this regard, streptomycin resembles the sulfonamides. Streptomycin is most effective against rapidly dividing cells, but is capable also of exerting some effect against organisms in the stationary phase.

One of the most serious drawbacks to the use of streptomycin as a chemotherapeutic agent is the frequency with which organisms resistant to its action emerge from otherwise sensitive strains, both *in vitro* and *in vivo*. The incidence of the emergence of organisms resistant to penicillin, on the other hand, is low. Indeed it is difficult to demonstrate that such resistant cells do appear. According to Demerec,[5] the streptomycin pattern of resistance differs from the penicillin pattern in that the streptomycin variants range from cells which are only slightly more resistant than the original bacterium to those having complete resistance. In the case of penicillin, the range is narrow.

Preliminary observations on the action of a new antifungal agent, Antibiotic XG, have been reported in a recent communication.[6] Antibiotic XG is an amphoteric polypeptide which exerts a remarkable inhibitory effect on the growth of many of the pathogenic fungi. It is both fungistatic and fungicidal, depending on the experimental conditions used. Its activity is influenced by the number of organisms present, the concentration of antibiotic, and the temperature of incubation. Its activity varies with the rate of multiplication of the organism. Present data on a single organism would suggest that this antimicrobial substance is ineffective against nonmultiplying cells. On standing in water, Antibiotic XG forms a hydrated gel which is biologically inactive.

Observations on the action of polymyxin D, an antimicrobial agent active in low concentrations against many of the Gram-negative microorganisms, have been reported by Bliss and her associates.[7] According to these investigators, the activity of polymyxin D is not influenced greatly by environmental factors; its activity is, however, influenced by changes in the size of inoculum. The action of polymyxin is inhibited by lipositol. Polymyxin D is bactericidal in action, and not merely bacteriostatic. It acts more rapidly than other known antimicrobial agents, especially when large amounts of the substance are used. It has been stated that such rapid action might suggest that it is not necessary for the bacteria to be dividing in order to be susceptible to the drug. According to Bliss and her coworkers, however, controlled experiments carried out at 37°C. and at 10°C. indicate that polymyxin acts only on the dividing cell. Polymyxin is of special interest in view of the fact that strains resistant to it fail to emerge in otherwise sensitive cultures.

Chloramphenicol and aureomycin, and to a less extent bacitracin, recently have come into widespread clinical use; yet little is known concerning the mechanisms by which they exert their antimicrobial effects. Bliss and Todd[5] have demonstrated that chloramphenicol and aureomycin are influenced in their activity by the number of cells present, as well as by the concentration of antimicrobial substance. Chloramphenicol exerts a slow but steady bactericidal effect against certain organisms, while aureomycin, at twice the minimal inhibitory concentration for *E. coli*, exerts only a bacteriostatic effect. It is believed that the bacteriostatic action of aureomycin is due to its instability in culture mediums, and that aureomycin may be capable of producing a bactericidal effect under conditions appropriate for maintaining stability of the product.

Little additional information on the mechanism of action of these or other antimicrobial agents exists. The technical difficulties encountered in determining reactions that occur within individual cells may account in part for the paucity of data. It is believed, however, that a gradual accumulation of such information, and its ultimate correlation, may result in a better understanding of the action of these compounds. Indeed, it may prove possible to interpret, from the mechanism by which an antimicrobial agent acts *in vitro*, its probable chemotherapeutic value.

In the present report, a few observations on the mechanism of action of polymyxins A and B and of neomycin will be discussed. These are reported at this time only to add to those data already available, for, as already stated, an accumulation of such isolated observations ultimately may lead to an understanding of the over-all mechanisms involved.

Materials and Methods

Throughout this study, partially purified preparations of polymyxins A and B (potencies 8500 and 6025 units per mg., respectively) and of neomycin (potency 180 units per mg.) were used. The preparations of polymyxins A and B were purified sufficiently to contain only polymyxins A and B respectively. All preparations of neomycin, on the other hand, contained more than one form of the antimicrobial agent in question. It has been assumed that differing forms of any given antimicrobial substance, although varying quantitatively in biological activity and slightly in chemical structure, will exert their antimicrobial effects by the same mechanisms. It is recognized, however, that this assumption may be erroneous.

The D/39/R strain of *D. pneumoniae*, the C203Mv strain of *Streptococcus hemolyticus*, the Oxford strain of *Staphylococcus aureus*, strains BW#41 and W#87 of *E. coli* (polymyxin-sensitive isolates from strains CN-1470 and ATCC39637 respectively), a freshly isolated strain of *Klebsiella pneumoniae*, and the H37Rv strain of *M. tuberculosis* were used for all studies. The specific organism used in each set of experiments varied according to the antimicrobial spectrum of the agent under test.

All observations were made by means of growth curves carried out in the usual manner. In the case of *D. pneumoniae*, *Streptococcus hemolyticus*, *Staphylococcus albus*, *E. coli*, and *K. pneumoniae*, a beef infusion agar at pH

7.8 and an incubation period of 24 to 48 hours were used. In the case of *M. tuberculosis*, the Dubos oleic acid-agar medium and an incubation period of 12 to 21 days were used. In the latter instance, deep colonies were observed and counts made with the aid of a Dazor Floating Magnifier, Model M 209-B.

Results

Polymyxin. *E. coli* (strain BW # 41) was used in all studies on polymyxins A and B. Results indicate that polymyxin A, like other antibiotics, may exert either a bactericidal or a bacteriostatic effect, depending on environmental factors. Bactericidal action may be observed with relatively low concentrations of the agent. In the presence of a small number of organisms (1,000 per cc.), a concentration equivalent to 0.9 μg. of pure polymyxin A HCl per cc. of medium may be sufficient to produce a bactericidal effect. The action of polymyxin A on large numbers of organisms, however, is generally bacteriostatic only (FIGURE 1). Presumably, concentrations of polymyxin A exist which are bacteriostatic against small numbers of organisms and which exert no effect against larger numbers. Such concentrations have not been observed in the experiments carried out to date. Indeed, in all instances, concentrations of polymyxin which were bacteriostatic against small numbers of organisms were also bacteriostatic against larger numbers, while concentrations which were bactericidal against small numbers of cells were either bactericidal or bacteriostatic against larger numbers. Concen-

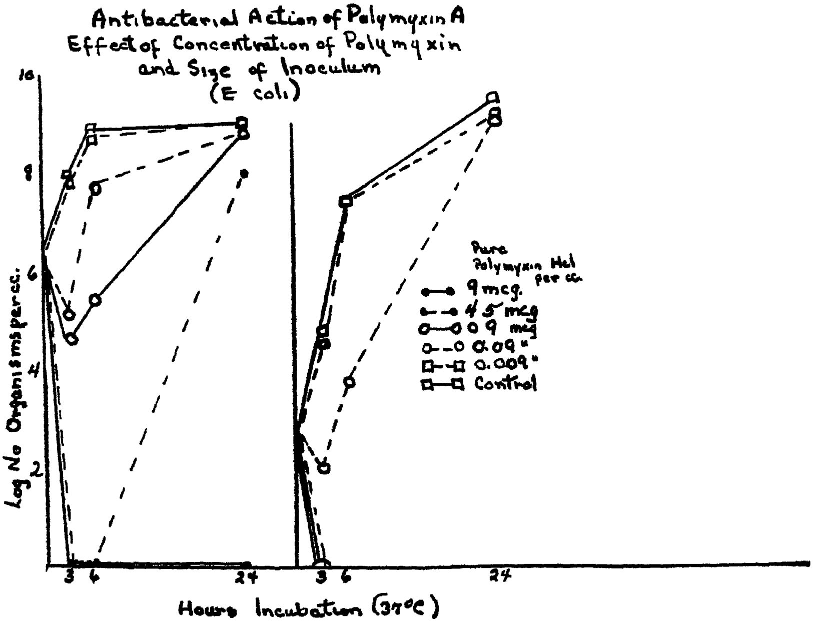

FIGURE 1

trations which showed no effect whatsoever against large numbers of organisms likewise were totally ineffective against smaller numbers of microorganisms. It is apparent therefore that the number of organisms present does not alter markedly the action of this antimicrobial agent.

Wide variations in the concentration of polymyxin A are necessary before an alteration in the effectiveness of the agent is observed. In the presence of concentrations of polymyxin A slightly below the bactericidal level, a sharp decrease in the activity of the drug may occur. This decrease in activity frequently is far in excess of that anticipated by the small change in concentration of antimicrobial substance. It is of interest that, in spite of the fact that wide variations in the number of organisms present are necessary in order to alter the effectiveness of a given concentration of polymyxin A, concentrations which at times may be bactericidal in action against a given number of cells may be only bacteriostatic on other occasions, even though other environmental factors presumably remain constant. The explanation for this is not readily apparent, for it is recognized that the incidence with which cells resistant to the action of polymyxin A emerge from otherwise sensitive strains is exceedingly low.

A limited series of observations would indicate that the bacteriostatic and bactericidal activity of polymyxin B is probably similar to that of polymyxin A. Both forms of polymyxin show a type of activity similar in most respects to that of polymyxin D, previously reported by Bliss and her associates. It has been stated by these investigators that polymyxin D is effective only against multiplying cells. In our experience, polymyxins A and B, although more effective against rapidly dividing cells, are capable of a bacteriostatic effect against cells which presumably have failed to multiply (FIGURE 2).

Neomycin. Neomycin, like the polymyxins and certain other antimicrobial agents, is both bactericidal and bacteriostatic, depending upon environmental factors. It is effective against both Gram-positive and Gram-negative microorganisms, and against the acid-fast bacilli. Available data suggest that the action of this substance may vary with the microorganism used. In the case of *K. pneumoniae* and *E. coli*, a 100-fold shift in the number of organisms present will result in a significant shift in the minimal concentration of neomycin necessary to effect complete sterilization or bacteriostasis of the culture (FIGURE 3). In the case of *Streptococcus hemolyticus* and *Staphylococcus aureus*, only a bacteriostatic effect has been observed even with low concentrations of the antimicrobial agent.

The action of neomycin on nonmultiplying cells is more striking than that with any other antimicrobial agent studied thus far (FIGURE 4). Indeed, neomycin may be strongly bactericidal against *K. pneumoniae* when the cells are in a stationary phase and show no evidence of multiplication. In the case of *Staphylococcus aureus* and *E. coli*, the action of neomycin on the nonmultiplying cell is bacteriostatic rather than bactericidal. Nonetheless, the effect is striking. In the case of *Streptococcus hemolyticus*, no bacteriostatic or bactericidal action has been demonstrated, except on the actively dividing cell.

The action of neomycin on certain species of microorganisms when in the stationary phase is of interest in view of the fact that interest in neomycin

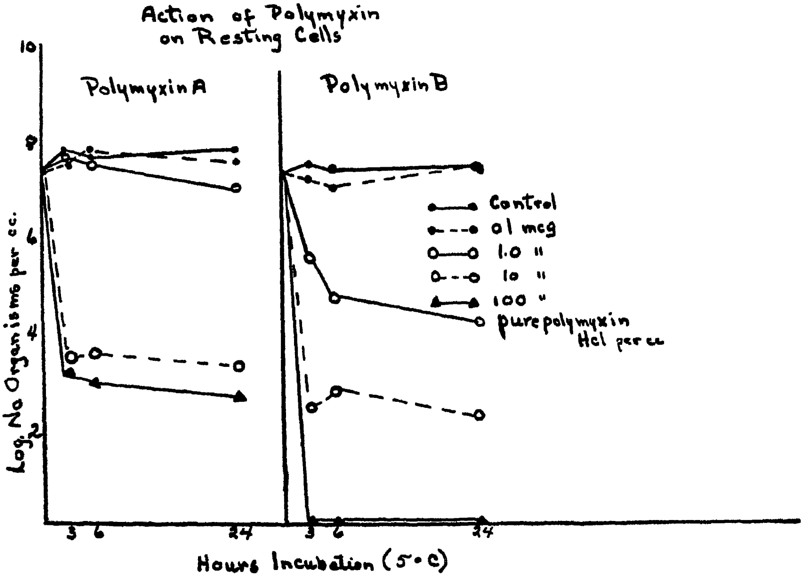

FIGURE 2.

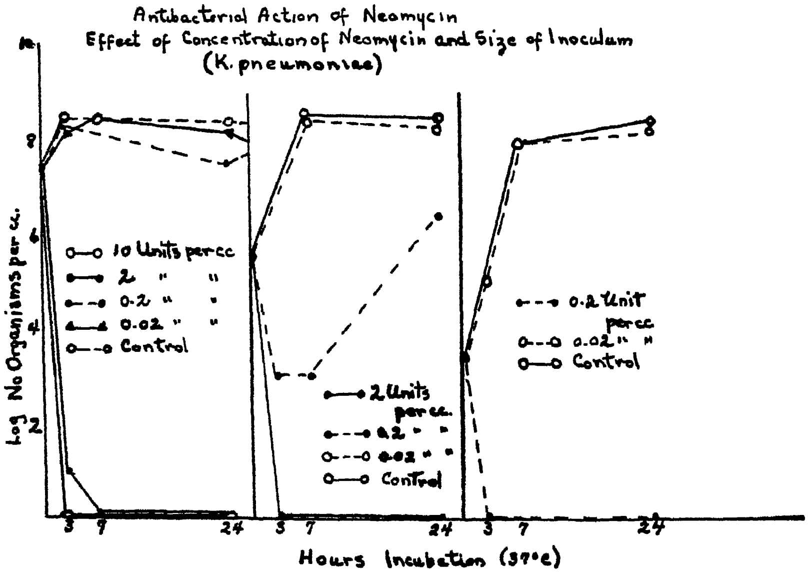

FIGURE 3.

as a potential chemotherapeutic agent has resulted primarily from the observation that it is active against both streptomycin-sensitive and streptomycin-resistant strains of *M. tuberculosis*. Although methods are not available which are sufficiently sensitive to prove conclusively that a single cell division has not occurred in the experiments described above, nevertheless, it is obvious that neomycin can exert a strong bacteriostatic or bactericidal effect in the absence of rapid cell multiplication.*

It is well recognized that tubercle bacilli divide at a slow rate, in comparison with most other bacteria, and it has been stated that streptomycin-resistant strains divide even more slowly than the sensitive parent strains. Studies of the action of neomycin on the H37Rv strain of *M. tuberculosis* have indicated that neomycin exerts only a weak bacteriostatic effect on this organism. Like streptomycin, it may prevent multiplication or slow the rate of growth of tubercle bacilli, but, in most instances, it is incapable of decreasing the number of cells to any great extent (FIGURES 5 and 6). Based on these *in vitro* data, it would seem probable that neomycin will exert only a suppressive effect on the course of tuberculous infections *in vivo*. This is in accord with the results of chemotherapeutic studies in mice which indicate that, although neomycin does exert such temporary suppression, infected animals treated with this agent for a limited period of time ultimately succumb with far advanced lesions.

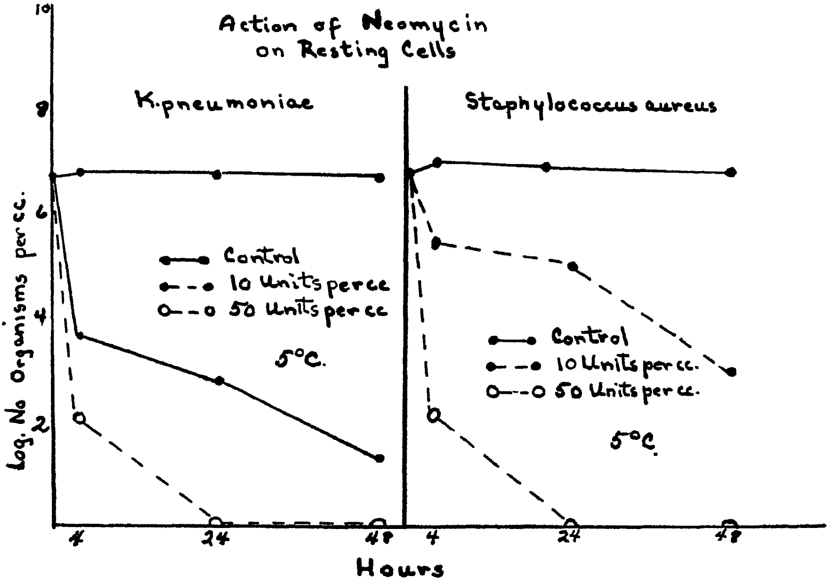

FIGURE 4.

Discussion

Several classifications of antimicrobial substances have been proposed in recent years. These agents may be divided, first, into two main groups:

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* That an impurity in the crude preparation of neomycin used is not responsible for the effect of this antimicrobial agent on stationary cells cannot be determined at present. It seems improbable, however, that an impurity possessing no antibacterial activity in itself could so influence a substance, such as neomycin, as to make it active against a type of cell against which, otherwise, it would have no activity.

(1) those of chemical origin and (2) those of biological origin. In any discussion of antibiotics, only those antimicrobial agents of biological origin concern us. These have been subdivided further by various investigators either on the basis of their solubility, their chemical structure, their toxicity, or on the basis of the type of organism by which they are produced. None of these classifications has served a useful purpose and none, therefore, has been entirely satisfactory.

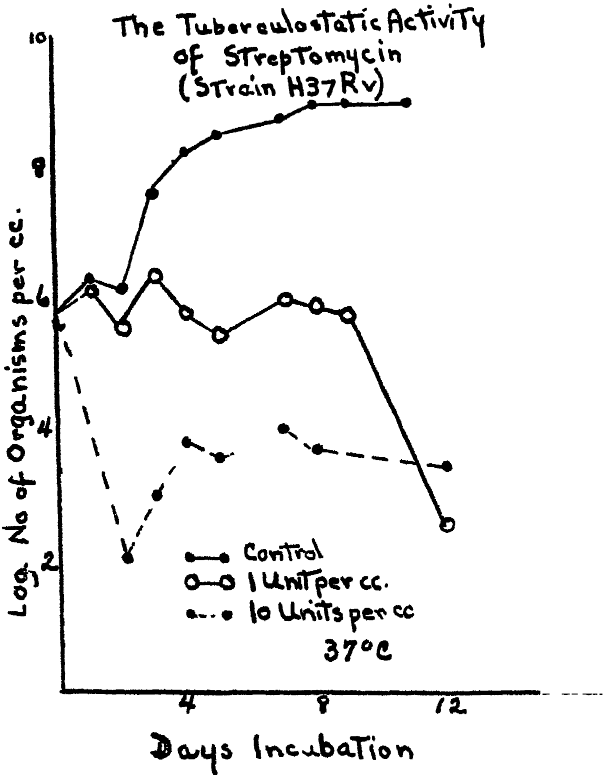

FIGURE 5.

To date, we know only that essentially all antimicrobial agents are alike in that they may exert either a bacteriostatic or bactericidal effect, depending upon the environmental conditions. The growth inhibitory action of each is influenced by the concentration of antimicrobial substance present. The number of cells and certain environmental factors will alter the activity of certain of the antimicrobial agents, and not others. Those so affected are generally ones which tend to be more bacteriostatic than bactericidal in action and permit a high incidence of resistant cells to emerge from otherwise sensitive cultures.

Each of the antimicrobial agents known is specific as regards the group of organisms against which it is effective. Some are more effective against the Gram-positive bacteria, some against the Gram-negative microorganisms, others against the acid-fast bacilli, and still others against the pathogenic fungi or the rickettsial group of organisms. There is no explanation at present, however, for the fact that the antimicrobial spectra of these various substances differ one from another. Furthermore, there is no explanation for the fact that certain antimicrobial substances, such as streptomycin and neomycin, permit the emergence of resistant cells from otherwise sensitive cultures with high frequency, while agents such as penicillin rarely, if ever, permit this to occur.

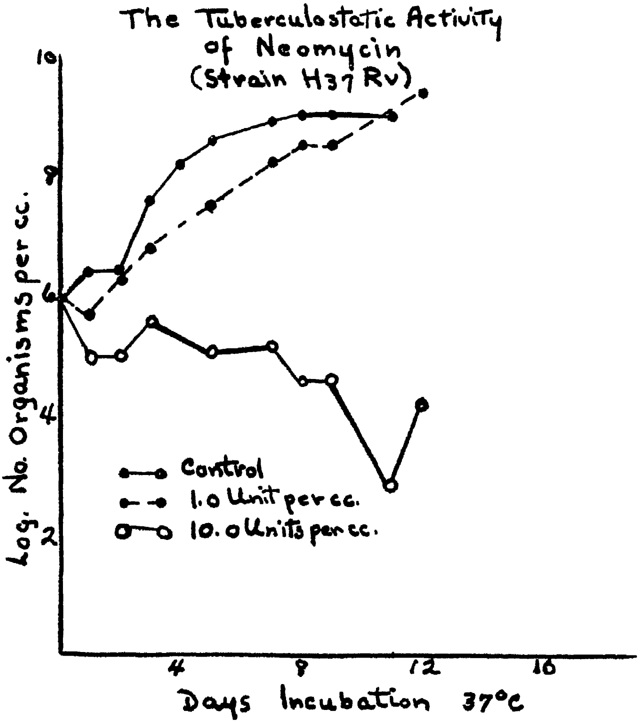

FIGURE 6.

It is apparent from the data reported herein that certain antimicrobial agents, such as penicillin, are active only against the actively dividing cell, but that many others, such as streptomycin, polymyxins A and B, and neomycin, may exert a definite effect on the stationary cell. The explanation for this is not readily apparent either. It has been suggested by Gale and his associates[9] that Gram-positive bacteria are able to assimilate glutamic acid from the medium in which they are grown and to concentrate the free amino acid within the bacterial cell. It has been suggested further that penicillin may be capable of blocking this assimilation of glutamic acid, and may exert its effect in this manner. Evidence has been brought forth by Maass and his associates[10] to support this thesis. Hunter and Baker,[11] using a different species of organism for their studies, have demonstrated recently, however, that a strain of *B. subtilis*, which produces no penicillinase, grows readily in a synthetic medium containing only ammonium sulfate as source of nitrogen and is incapable of elaborating significant amounts of amino acids into the basal medium during growth. It is as sensitive to penicillin when tested in the basal medium as when tested in a "complete" tryptose phosphate medium. It is apparent, therefore, that in this instance, penicillin must inhibit growth by some mechanism other than interference with the assimilation of preformed amino acids by the bacterial cell. Whether or not this proves to be so with other organisms remains to be seen.

That streptomycin probably interferes with respiration has been demonstrated by Umbreit.[12] The question thus arises: do those antimicrobial agents which are capable of acting on stationary as well as on actively multiplying cells function by virtue of their ability to interfere with respiration, while those that act only on the dividing cell function by some other mechanism, such as, perhaps, interference with assimilation of an essential foodstuff? There is no answer to this at present, but it is hoped that future studies will provide the necessary information.

The antimicrobial agents of biologic origin, at present, may be divided loosely into several groups. Among them, one group comprises those agents which are derived from bacteria, are polypeptide in nature, are thermostable, have a high molecular weight, diffuse slowly, and are absorbed and excreted at a relatively slow rate. These generally exhibit a high degree of toxicity which manifests itself by hemolysis, precipitation of proteins, or by impairment of renal function. One may include in this group the polymyxins, gramicidin, bacitracin, Antibiotic XG, circulin, and subtilin, among others. It is possible that not all of the polypeptide antimicrobial agents are toxic. Nonetheless, the high molecular weight of these substances suggests that they probably will diffuse poorly in the body, and to date all have been essentially toxic compounds. Probably due to their stability, these are the most frequently observed and most readily isolated antimicrobial agents.

Other compounds, including some that are strongly acidic in nature and some that have been only poorly characterized, also exhibit a high degree of toxicity. These are of little or no value chemotherapeutically and have received little attention.

Another group, of some interest at present, includes those agents which are derived from fungi and are basic in nature but of smaller molecular size than the basic polypeptides. These exhibit a lower degree of toxicity and comprise a relatively useful group of agents. In this group, one may include streptomycin and probably neomycin.

Penicillin, probably the most widely studied antibacterial agent, and possibly mycomycin, a recently described tuberculostatic agent, stand apart from these other agents in that they are weakly acidic in nature, form readily water-soluble salts, are rapidly absorbed and excreted, and exhibit little or no toxicity. Penicillin, as previously stated, approaches, more nearly than any other agent known, the ideal chemotherapeutic agent. It is conceivable that it is the group of weakly acidic compounds which will compare favorably with this substance.

It should be emphasized that to date only aureomycin and chloramphenicol have shown antirickettsial activity.* Aureomycin is an amphoteric base while chloramphenicol is a neutral substance. The ideal antirickettsial and antiviral agents, when ultimately isolated, may differ widely in chemical structure from the ideal antibacterial agents.

It is only by more extensive study of these known antimicrobial substances, both toxic and non-toxic, that an understanding of which mechanisms of action permit most satisfactory chemotherapeutic activity will result. Only by extensive studies designed to isolate and investigate large numbers of both the stable and less stable antimicrobial agents will it be possible to accumulate sufficient data to permit an evaluation of their individual mechanisms and a correlation of their activity with their therapeutic efficiency.

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*Subsequent studies have indicated that terramycin, a newly discovered antimicrobial agent, also possesses antirickettsial properties. Terramycin is an amphoteric compound which reacts with acids and bases to form water-soluble salts.

THE EFFECT OF SURFACE-ACTIVE AGENTS ON ANTIBIOTICS: AN INFORMAL REPORT*

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The observation that traces of green soap, remaining in laboratory glassware after washing, might account for irregularities in titrations with polymyxin D led to an investigation of the effects of surface-active agents on antibiotics in general.

The method was to make serial twofold dilutions of the antibiotic in heart infusion broth (pH 7.2) containing the surface-active agent. The tubes were inoculated with a strain of *E. coli*, about 200,000 organisms, and growth, as shown by visible turbidity, was read after 18–20 hours at 37 C.

The results, expressed as the ratio between minimal inhibitory concentration of drug in the presence of the agent and minimal inhibitory concentration in plain broth, are shown in TABLE 1. From this presentation of the data, one receives the impression that the antibiotics derived from spore-bearing bacilli—the polymyxins and circulin—are particularly sensitive to surface-active agents. Soap and Aso-lectin were antagonistic, raising the titration end points of the polymyxins and circulin over 1000 times. Lipositol, tested only against polymyxin D, also was antagonistic, but to a lower degree. Tween 80 had the reverse effect, causing a fourfold *increase* in activity. End points of aureomycin, chloromycetin, and streptomycin were much less affected. Soap, in fact, was almost inert against the last two antibiotics. Penicillin end points were not altered by soap, Aso-lectin, or Tween 80 in the concentrations tested.

The possibility that the alkalinity of broth solutions of soap and Aso-lectin might be responsible for their antagonistic action was not tenable, because such solutions had a much greater effect than broth adjusted to a higher pH with NaOH. Polymyxin D, as well as polymyxin B, circulin, and aureomycin, deteriorates in an alkaline environment. The fact that its end point on this occasion was the same at pH 8.6 as at pH 7.2 presumably reflects the rapidity with which it kills *E. coli.* The organisms were dead before deterioration could occur. On the other hand, the increased activity of streptomycin and chloromycetin in an alkaline medium might explain their lack of response to soap.

"Letheen" broth, which has been recommended for the neutralization of cationic antiseptics, failed to antagonize any of the antibiotics in the present series. Apparently, its content of Tween 80—5000 μg./ml.—was sufficient to offset the antagonistic action of the 700 μg./ml. of Aso-lectin present in this medium.

An inverse relationship was noted between the efficacy of the antibiotics in inhibiting strains of *Proteus* and their sensitivity to soap and Aso-lectin. Moreover, a filtrate of a broth culture of a strain of *Proteus* decreased the antibacterial activity of polymyxin D but not of chloromycetin, while a staphylococcal filtrate had no effect on polymyxin.

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The special sensitivity of the polymyxins and circulin to antagonism by soap and Aso-lectin disappears when the data are presented on a quantitative basis, as in TABLE 2. There, it is seen that nearly as much aureomycin as polymyxin was antagonized by a given amount of soap, and as much chloromycetin and streptomycin was antagonized by Aso-lectin. The difference is still further reduced when the data are converted into mols of drug inactivated, since the polymyxins and circulin have about twice the molecular weight of aureomycin and streptomycin and four times that of chloromycetin.

TABLE 1

EFFECT OF SURFACE-ACTIVE AGENTS ON TITRATION END POINTS OF ANTIBIOTICS

Agent	Concentration µg./ml.	*Antibiotic*						
		Polymyxin		*Circulin*	*Aureomycin*	*Chloromycetin*	*Streptomycin*	*Penicillin G*
		D	*B*					
		(*minimal inhibitory concentration for* E. coli—*µg./ml.*)						
		.2	.5	.6	6	5–10	3–6	50
		ratio of m.i.c. in presence of agent to m.i.c. in HIB pH 7.2						
HIB pH 8	—	1	4	8	16	.5	.125	
Soap (pH 7.4–7.8)	8	2	1	2	2	1	1	
	250	16	32	64	4	1	1	
	500	:	:	:	:	:	:	1
	5000	5120	1024	2048	64	4	2	
Aso-lectin (pH 7.4–7.6)	8	1	2	4	1	1	1	
	250	32	32	16	1	4	1	
	700	:	:	:	:	:	:	1
	5000	1280	1280	2560	32	50	100	
Lipositol	8	1						
	250	8						
	5000	128						
Tween 80	5000	.25	.25	.25	.5	1	1	1
"Letheen" HIB	—	.5	.5	.5	1	1	.5	
Proteus Filtrate (undiluted)		4				1		
		m.i.c. for strains of Proteus—µg./ml.						
		>100	>100	>100	5–100	2–25	0.6–12	

Valko and DuBois[1] reported that the bactericidal effect of surface-active cations could be reversed by treatment with anionic agents even after a 10-minute exposure of the bacteria to the cation. After an exposure of 30 minutes, recovery did not occur. The reversibility of polymyxin D's effect on *E. coli* was tested by adding soap to cultures at intervals during a growth study. The results are shown in TABLE 3. Polymyxin, at a concentration of 0.2 µg./ml., was rapidly bactericidal for *E. coli*. This action was abolished by the presence of 100 µg./ml. of green soap, introduced before inoculation of the tubes. The culture grew at the same rate as the broth controls (with or without soap). A delay of 10 minutes in the addition of soap resulted in a retardation of growth. When the soap was not added until after the organisms had been exposed for 30 minutes to polymyxin, the culture nearly failed to recover. The population declined for the next 30 minutes at a rate that was even faster than that in the tube containing polymyxin alone, but, two hours later, multiplication had begun, and, by 24 hours, growth was equal to that in the controls. It has been observed repeatedly that a 60-minute delay in the addition of the antagonist is fatal. The recovery from exposure to polymyxin D that occurred in the presence of soap represents, it is believed, not a resuscitation of "dead" bacteria but merely the multipli-

Table 2

Amount of Antibiotic Inactivated by Soap and Asolectin

Agent	*Amount*	*Antibiotic*					
		Polymyxin *D*	*Polymyxin* *B*	*Circulin*	*Aureomycin*	*Chloromycetin*	*Streptomycin*
		micrograms					
Soap	8	.08	0	.39	3.1	0	0
	250	1.2	12.1	25	9.4	0	0
	5000	400	400	800	197	9.4	4.7
Aso-lectin	8	0	.16	.94	0	0	0
	250	2.4	5.0	4.7	0	7.5	0
	5000	100	200	800	97	200	200

Table 3

The Effect of Soap on the Bactericidal Action of Polymyxin D

(0.2 µg./ml. polymyxin; 100 µg./ml. soap added at different times; organism: *E. coli*)

Time of plating (after inoc.)	*Polymyxin*				*Broth*	
	Control	*Soap added*			*Control*	*Plus soap at start*
		at start	*at 10 min.*	*at 30 min.*		
	(bacteria per ml.)					
0	270,000	180,000	(200,000)[1]	(200,000)	160,000	(200,000)
10 min.	4,720	160,000	(5,000)	(5,000)	160,000	(200,000)
30 min.	2,640	350,000	120,000	710	370,000	260,000
60 min.	220	490,000	196,000	40	410,000	310,000
3 hr.	<10	14,400,000	1,760,000	2,800	18,000,000	18,000,000
24 hr.	–	++++	++++	++++	++++	++++
48 hr.	–	++++	++++	++++	++++	++++

***Figures in parentheses are estimates of counts based on analogy with corresponding plated cultures**

cation of the remaining viable organisms. To test this point, plates were poured immediately before and immediately after the addition of soap, following 10-, 30-, and 60-minute exposures to 0.2 μg./ml. solutions of polymyxin D. If resuscitation occurred, one would expect a higher count in the second plate than in the first. In all three instances, the reverse was noted: the second plates showed significantly lower counts than the first, indicating that no bacteria had been liberated from the harmful effect of the drug. Instead, more bacteria had succumbed during the 2-minute interval between the platings.

No conclusions regarding the mode of action of this group of antibiotics can be drawn from the experiments cited here. Indeed, further work must be done before anything concrete can be said regarding the mechanism of the observed interference by surface-active agents. In certain respects, the findings are puzzling. One might reasonably expect that the response to alkali or surface-active agents would accord with the acidic or basic nature of the antibiotic. The polymyxins, circulin, aureomycin, and streptomycin are salts of bases, chloromycetin is neutral, and penicillin is an acid salt. Yet, the first four antibiotics are less active at a high pH, differing in this respect from streptomycin as well as from other cationic antiseptics, such as quaternary ammonium salts and the diamidines. Furthermore, streptomycin resembles chloromycetin, rather than polymyxin, in its insensitiveness to soap. Another area in which the anticipated pattern is infringed is in the behavior of the antibiotics in the presence of serum. Serum interferes strongly with the antibacterial activity of aureomycin and penicillin, but has a negligible effect on the polymyxins, circulin, streptomycin, and chloromycetin. The explanation of such anomalies lies, no doubt, in the fact that a number of influences are at work. Oversimplification of the problem or undue emphasis on any single aspect can only lead to confusion'

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STUDIES ON THE SYNERGISM BETWEEN BACITRACIN AND PENICILLIN: CORRELATION OF *IN VIVO* WITH *IN VITRO* RESULTS*

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The investigation of possible synergistic action of mixtures of antibiotics is not only of interest to the microbiologist from a purely scientific point of view but is of practical importance in providing more effective means of combating infections. The synergistic action of mixtures of penicillin and bacitracin was first observed by Eagle and his associates[1, 2] on the Reiter strain of the spirochete of rabbit syphilis. This very dramatic synergistic effect was present both *in vitro* and *in vivo.* Bachman[3] has reported additive and synergistic action by such a mixture on alpha and gamma streptococci. In 1948, this laboratory made preliminary reports to the Office of the Surgeon General on the effect of the combined action of penicillin and bacitracin on coagulase-positive staphylococci, the beta, alpha, and gamma streptococci and *C. welchii.* This paper will present further details with regard to this phenomenon.

The strains of *Staphylococcus aureus* used in this study were all freshly isolated and include both penicillin-sensitive and penicillin-resistant strains. Some of the strains are known to have become resistant during the course of penicillin therapy. Others were resistant on primary isolation from the lesion before penicillin therapy was instituted. These lesions included cases of synergistic gangrene, acute and chronic osteomyelitis, bacterial endocarditis, meningitis, brain abscesses, chronic fibrotic bronchiectasis in children, as well as minor surgical infections. Strains were also included which showed an increase in resistance to bacitracin, following systemic or aerosol therapy. The alpha and gamma hemolytic streptococci were obtained from cases of endocarditis or pyelitis, while the beta hemolytic streptococci were isolated from cases of cellulitis and from infected burns. The Welch strains were recovered in 1942—1945 from severe civilian accidental wounds and burns.

Methods of Testing Synergism

Three different experimental procedures were used in testing for the possible additive or synergistic action between penicillin and bacitracin on strains of staphylococci. The first was the observation of complete inhibition of growth in beef heart infusion broth containing 1 per cent tryptose and 1 per cent proteose peptone, by a combination of fractional portions of the minimal completely inhibitory concentration, when each antibiotic was used alone. The second method involved colony counts in beef heart infusion agar pour plates containing the antibiotics alone and together. The third method was to determine, by interval sampling, the differences in the rate of killing when the antibiotics were used separately and in combination.

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* The commercial bacitracin used in these studies was supplied by the Commercial Solvents Corporation of Terre Haute, Indiana.

In all tests, crystalline penicillin G, dissolved to a concentration of 2,000 units per ml. in sterile distilled water, was used. Lyophilized bacitracin was dissolved in sterile physiological saline to 1,000 units per ml. These stock solutions were then diluted in beef heart infusion broth to the desired concentrations.

Serial Dilution Test. The final volume in the serial dilution sensitivity tests was 2 ml. The inoculum used to seed the tubes was 0.1 ml. of a 10^{-4} dilution of a 5-hour culture of the test strain of staphylococcus. Eighteen-hour cultures in blood broth were used when the test strain was a streptococcus. Readings were made after 24, 48, and 72 hours of incubation at 37.5°C. The end-point or minimal inhibitory concentration was absence of visible turbidity.

In the tests with *C. welchii*, 0.1 per cent of agar had been added to beef heart proteose peptone infusion broth. The medium was tubed deep in 4.5 ml. amounts. It was boiled for five minutes and then brought rapidly to 45°C. Four-tenths ml. containing the concentration of each antibiotic desired for the test was added to the tubed media, the tube was rotated, and 0.1 ml. of a 10^{-2} dilution of an 18-hour culture of the organism in 1 per cent dextrose broth was added. The tube was again rotated and then placed in an anaerobic jar for incubation at 37.5°C. Readings were made after 24, 48, and 72 hours of incubation.

Pour Plate Method. In preparing the pour plates, tubes containing 23 ml. of 2 per cent beef heart infusion agar were heated until the agar was melted. They were then cooled to 45°C. One ml. of the beef heart infusion broth, containing the unitage of penicillin or bacitracin, or penicillin and bacitracin calculated to give the required concentration in the final 25 ml. volume, was then added. The tubes were rotated and then seeded with 1 ml. of a 10^{-4} dilution of a 5-hour culture of the test strain of staphylococcus. All tubes were rotated again to insure even distribution of the antibiotic and inoculum, and the agar was then poured into Petri dishes. A control plate without the addition of the antibiotics was similarly prepared. The concentrations of antibiotic in the pour plates were based on the minimal concentration previously found necessary for complete inhibition of growth of the test strain in the beef heart infusion broth. The series of agar plates contained twice, once, two-fifths, one-fifth, and one-tenth of this concentration for bacitracin and penicillin alone, and combinations of one-fifth or one-tenth of each antibiotic.

Interval Sampling. The effect of mixtures of penicillin and bacitracin on the rate of killing of a *Staphylococcus aureus* strain was tested as follows: A series of four tubes containing 8 ml. of broth was seeded with 1 ml. of a 10^{-4} dilution of a 5-hour broth culture of the staphylococcus. To each tube in order was added 1 ml. of a solution containing penicillin in a decreasing amount, calculated to give in the final mixture twice, once, one-half, two-fifths, and one-fifth of the previously determined minimal complete inhibitory concentration. A similar series was set up with bacitracin. A third series of three tubes was set up, with a final concentration in the broth of one-half plus one-half, one-fifth plus one-fifth, and one-tenth plus one-tenth of the concentrations of each antibiotic previously found to inhibit completely growth in the broth. A tube with 9 ml. of broth was seeded with 1 ml. of 10^{-4} dilution of the culture as a control for the growth of the staphylococci in the absence of any antibiotic. The tubes were incubated in a water bath at 37.5°C., and, at the desired intervals, samples were withdrawn. Agar pour plates were then made from suitable dilutions of these samples. The plates were incubated for forty-eight hours, and colony counts were then made.

Results Obtained with Staphylococci

Broth Dilution Method. Sixteen strains of coagulase-positive *Staphylococcus aureus* with a penicillin resistance varying from 1 to 32 units per ml. were tested. All of these strains were isolated from cases which had failed to respond to penicillin therapy. Eight were from chronic infections which were cultured after penicillin therapy had been concluded, and the original sensitivity of these strains is not known. Six were from strains which developed resistance as a result of prolonged penicillin therapy, and two were isolated within one to two days after the institution of this therapy.

TABLE 1

SYNERGISTIC ACTION *in vitro* BETWEEN PENICILLIN AND BACITRACIN

Response to previous penicillin therapy	*Number of Strains*	*Sensitivity in u./ml.*		*Synergistic action* in vitro *demonstrated in*
		Pen.	*Bac.*	
Poor	16	0.375–32	0.05–3.0	12
Good	14	0.01 – 0.5	0.01–5.0	10

Of these sixteen strains, only two failed to show complete inhibition of growth when one-fifth of the minimal complete inhibitory concentration of penicillin was combined with one-fifth of the minimal complete inhibitory concentration of bacitracin. The growth of one strain was prevented by a thirty-second of the minimal inhibitory concentration of penicillin added to one-tenth of the minimal inhibitory concentration of bacitracin. However, it must be pointed out that the 24-hour reading on the penicillin sensitivity of this case was 8 units per ml., which jumped to 32 units per ml. after 72 hours. The end point for bacitracin sensitivity, namely, 1.25 units per ml., remained the same during this period.

Fourteen strains were from infections which had responded to penicillin therapy or represented isolations before penicillin therapy was instituted. Of these strains, ten were completely inhibited when one-fifth of the minimal inhibitory concentrations of each antibiotic were combined. With the other four strains, an additive but not a clear-cut synergistic action was demonstrated. These results are shown in TABLE 1.

Agar Pour Plate Method. Three strains were tested for inhibition of growth in agar pour plates containing penicillin or bacitracin or a combination of fractional doses of both antibiotics. These strains had been inhibited by one-fifth of the minimal inhibitory concentration of penicillin plus one-fifth of the minimal inhibitory concentration of bacitracin in the broth tubes. The synergistic action of the combination of antibiotics is evident in TABLE 2.

Interval Sampling Method. The rate of killing of staphylococci by means of varying combinations of penicillin and bacitracin is shown in TABLE 3. It will be seen that the mixture of one-fifth of the minimal completely inhibitory concentrations of the two antibiotics resulted in almost complete sterilization of the experimental set-up, whereas two-fifths of each of the antibiotics alone permitted multiplication. The combination of one-tenth of the previously found minimal inhibitory concentration for each antibiotic was bacteriostatic over the 6-hour period, with some evidence of reduction in number of viable cocci, but at the end of 24 hours the cocci had grown out in countless numbers. (TABLE 3.)

TABLE 2

EFFECT OF COMBINED ACTION OF PENICILLIN AND BACITRACIN IN AGAR POUR PLATES ON *Staphylococcus aureus*

Strain	**M.I.C in u./ml.*		*Colony Count*					
			*2/5 *M.I.C.*		*1/5 *M.I.C.*		*1/5 *M.I.C. Pen plus 1/5 *M.I.C. Bac.*	*1/10 *M.I.C. Pen plus 1/10 *M.I.C. Bac.*
	Pen.	*Bac*	*Pen.*	*Bac.*	*Pen.*	*Bac*		
Ca	1.5	1.5	+++	+++	++++	++++	2	310**
Sch	0.5	0.625	+++	++	++++	++++	5	128**
Mo	0.09	0.25	++	+++	++++	++++	1	+

* M.I.C.—No colonies developing in pour plates

** Total number of colonies on three pour plates.

TABLE 3

ACTION OF MIXTURES OF PENICILLIN AND BACITRACIN ON *Staphylococcus aureus* (SCHWARTZ) VIABLE ORGANISMS IN THOUSANDS PER ML

Hours	*Control*	**Penicillin 0.15 u./ml.*	**Bacitracin 0.2 u./ml.*	***Penicillin 0.075 u./ml.*	***Bacitracin 0.1 u./ml.*	***Penicillin 0.075 u./ml plus **Bacitracin 0.1 u./ml.*	****Penicillin 0.0375 u./ml. plus ***Bacitracin 0.05 u./ml.*
0	5.2						
1	8.3	6.4	3.8	7.1	4.3	4.4	4.7
2½	21.6	8	3.6	43	19.9	0.9	1.8
4	+	6	5	128	49	0.1	0.9
6	+	25.9	20.1	+	+	0.01	1.1
24	+	++++	++++	+	+	0.001	+

* 2/5 minimal inhibitory concentration

** 1/5 minimal inhibitory concentration.

*** 1/10 minimal inhibitory concentration.

+ Too many organisms to count in pour plates made with 1 cc. of 10^{-3} dilutions of tubes after 6 hours' incubation, in 10^{-6} dilutions after 24 hours' incubation.

The minimal concentrations of penicillin and bacitracin found to be completely inhibitory over a 72-hour incubation period proved to be bactericidal as well under the conditions of these experiments. One-half of such concentrations were bacteriostatic over the 6-hour test period, but, at the end of 24 hours, multiplication had taken place. A combination of these concentrations ($\frac{1}{2} + \frac{1}{2}$) was bactericidal.

The lethal action of bacitracin on strains of coagulase-positive staphylococci has been repeatedly demonstrated by periodic sampling of the cultures containing varying quantities of the antibiotic. This is shown in TABLE 4. It will be seen that, at completely bacteriostatic concentrations, bacitracin is also bactericidal. There is also evidence that, at concentrations which are not bactericidal, bacteriostasis with some reduction in the numbers of viable organisms may persist for as long as six hours. There has been no evidence of preliminary multiplication before bactericidal action begins.

TABLE 4

EFFECT OF CONCENTRATION OF BACITRACIN ON RATE OF KILLING OF *Staphylococcus aureus* (WATSON) VIABLE ORGANISMS IN THOUSANDS PER ML

Age of Culture	*Hours*	*Concentration of Bacitracin in Units per ml*				
		10	3	1.5	0.75	0
5 Hours	0	30	29	28	25	36
	2	3.9	2.6	7	12	150
	4	0	0.2	0.3	13.8	++
	6	0.01	0.03	0.18	16.6	++++
	8	0	0	0.6	+++	++++
	10	0	0	0.01	++++	++++
	24	0	0	0	++++	++++

TABLE 5

COACTION OF PENICILLIN AND BACITRACIN ON ALPHA AND GAMMA STREPTOCOCCI

Strain	**M.I.C. in u./ml.*		*Fractions of M.I.C. giving complete inhibition with*	
	Pen.	*Bac.*	*Pen. + Bac.*	
H	8	1.25	1/2	1/2
W	4	0.75	1/3	1/3
L	5	1	1/5	1/4
J	8	0.75	1/2	1/3
Le	5	1.25	1/2	1/2

* M.I.C.—Minimal concentration necessary for complete inhibition of growth.

Results Obtained with Streptococci

When mixtures of bacitracin and penicillin were tested for their coaction on the streptococci, the synergistic action on the alpha and gamma strains was not as striking as on the staphylococci. Five strains were tested, four from cases of subacute endocarditis and one from a case of pyelitis. These cases had failed to respond to large doses of penicillin, and the strains showed a high resistance *in vitro* at the time the tests for coaction were run. The results of these tests are given in TABLE 5.

These final 72-hour readings show an additive rather than a clear-cut synergistic action in four of the five strains. Bachman,[3] using the turbidimetric method, reported more striking results from the combination of bacitracin and penicillin on such streptococci. His results are based on 24-hour readings, while those of the authors represent 72-hour readings. It may well be that the 24-hour readings are a more reliable guide to clinical effectiveness, since at 37.5°C. the half-life of bacitracin is 24 to 36 hours.

With the exception of certain strains of Group A hemolytic streptococci, the beta hemolytic strains have shown only additive action by penicillin and bacitracin. Observation of inhibition of growth, however, over the first 24 hours of incubation at 37.5°C. showed a definitely prolonged bacteriostatic action when fractional doses ($\frac{1}{5}$ to $\frac{1}{10}$) of the minimal inhibitory concentration of bacitracin and penicillin were added to the broth set-ups, even though there was visible multiplication after a 72-hour period of incubation. There was, however, a definite synergistic action on four of the eight strains of Group A streptococcus tested, as shown by absence of growth when one-fifth of the minimal inhibitory concentrations of each antibiotic was added to the broth.

TABLE 6
COACTION OF PENICILLIN AND BACITRACIN ON *Clostridium welchii*

Strain	**M.I.C. in u./ml.*		*Fractions of M.I.C. giving complete inhibition with*	
	Pen.	*Bac.*	*Pen. + Bac.*	
Zu	0.08	0.01	1/5	1/10
H	0.05	0.003	1/5	1/5
B	0.02	0.006	1/10	1/5
Mc	0.04	0.006	1/5	1/5
S	0.08	0.008	Growth in 1/5	1/5

* M.I.C.—Minimal concentration necessary for complete inhibition of growth.

Results Obtained with Clostridium welchii

When the technic previously described under "Methods of Testing Synergism" was used, there was clear-cut evidence of a synergistic action by penicillin and bacitracin on four of the five strains tested. These results are shown in TABLE 6.

Correlation of in vivo *with* in vitro *Results*

It is difficult to be certain of the synergistic action of the antibiotics *in vivo*, in treating clinical infections. "Synergism" means, in effect, that two agents can do something together that neither of them can do separately. Clinical proof of such effect might be thought to entail the clinical trial of first one and then the other of the agents, with failure of both and success following a combination of the two. Failure with one and success following the addition of the second might indicate synergism, although such a result might not preclude the success of the second agent, if it had been used first.

When penicillin fails to bring an infection under control and the addition of bacitracin to the therapy promptly effects a cure, and when the examination of the culture reveals that the organism is resistant to penicillin while it is susceptible to a combination of the two drugs in relatively small fractions of the completely inhibiting concentration of each, we have some indication that the synergism is at work *in vivo*. Such a situation is illustrated by the following cases:

A boy of 13, with all of the clinical signs and symptoms of acute osteomyelitis of the femur, was treated within twelve hours of the onset of symptoms with 100,000 units of penicillin, followed by 50,000 units every three hours. With no improvement over the first twelve hours, the dose was doubled. There was slight but not striking improvement during the first three days, and symptoms and signs returned on the fourth day, when the blood culture revealed the cause of the infection to be a hemolytic *Staphylococcus aureus*. The absence of the expected response to penicillin suggested the addition of intramuscular bacitracin, and it was given in a dosage of 10,000 units every six hours. Thereupon, the symptoms and signs promptly disappeared and the boy went on to rapid and complete recovery without a breakdown of the bone.

Sensitivity studies of the organism revealed that it required 32 units/ml. of penicillin or 1.25 units/ml. of bacitracin to inhibit growth *in vitro*. However, there was complete inhibition of growth by one thirty-second of this concentration of penicillin plus one-tenth of this concentration of bacitracin.

A six-weeks-old baby with a hemolytic *Staphylococcus aureus* septicemia and meningitis, resulting from an abscess of the face of sinus origin, had metastatic foci in the lungs and in the subcutaneous tissues. Penicillin, streptomycin, sulfadiazine, and aureomycin all failed to bring the infection under control, but when bacitracin was added to the treatment, both by intramuscular and intrathecal injection, the baby promptly came out of the coma into which she had fallen, and, on the fourth day of bacitracin treatment, the blood culture and spinal fluid became negative. Thereafter, she went on to a rapid and complete recovery.

Laboratory studies of the blood culture revealed some variation in susceptibility to both penicillin and bacitracin, some colonies being sensitive to 0.75 units/ml., while others required 10 units/ml. of penicillin for complete inhibition. Some colonies were sensitive to 0.375 units/ml. of bacitracin, while others required 3 units/ml. It was found, however, that those most resistant to penicillin proved to be the most sensitive to bacitracin, and synergism tests revealed that the most resistant staphylococci were inhibited by one-fifth of the complete inhibiting concentration of bacitracin plus one-tenth of the complete inhibiting concentration of penicillin, while the most sensitive colonies were inhibited by one-tenth of the complete inhibiting concentration of bacitracin plus one-fifth of the complete inhibiting concentration of penicillin. This seemed to confirm the clinical impression that the synergistic action of these antibiotics played a significant role in the recovery of the child from the infection.

The third patient was a football player, aged 20. Three days before admission, after a trivial abrasion of the leg in football scrimmage, he developed, very rapidly, a massive swelling of the right leg with tenderness all over the tibia, clearly indicating an acute osteomyelitis. He was put to bed and given 300,000 units of penicillin every three hours, but without any improvement in his toxicity or fall in his temperature over a period of three days. On the fourth day, he was, therefore, given intramuscular injections of 20,000 units of bacitracin every eight hours in addition to penicillin. This was followed by a prompt resolution of the swelling and a fall of temperature to normal within forty-eight hours, so that he was able to leave the hospital on the sixth day after admission and even felt well enough to attend a football game.

Sensitivity tests of the organism showed that it was relatively resistant to both penicillin and bacitracin alone, requiring 1.5 units/ml. of each for complete inhibition. Inasmuch as staphylococci are usually more sensitive to penicillin than bacitracin, unit for unit, this indicates a relative sensitivity to bacitracin. When one-fifth of each of these inhibiting doses were combined, they successfully prevented the growth of the organism *in vitro.* It would seem, therefore, that this case presents evidence of the synergistic action of these two antibiotics in the control of this infection.

Summary

1. Bacitracin combined with penicillin had a definite synergistic action *in vitro* on twenty-four of the thirty strains of coagulase-positive hemolytic *Staphylococcus aureus* tested.

2. This synergistic action was exerted on strains with a high resistance to these antibiotics as well as on those with sensitivities in the "average" range.

3. In the authors' experiments, the coaction of bacitracin and penicillin on the strains of alpha and gamma streptococci tested was additive only. A synergistic effect was exerted on four of the eight strains of Group A beta hemolytic streptococcus tested.

4. When a synergistic action between bacitracin and penicillin had been demonstrated *in vitro* on the organism responsible for the infection, patients who had failed to respond to penicillin alone made an excellent response to combined penicillin and bacitracin therapy.

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AN APPROACH TO A CORRELATION BETWEEN *IN VIVO* AND *IN VITRO* ACTIVITIES OF ANTITUBERCULAR COMPOUNDS

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During the past five years, there has been an invigorating upsurge in the attack on the problem of the chemotherapy of tuberculosis. This was probably due in part to the remarkable activity of streptomycin in this disease and, in part, to the gradual and relatively satisfactory solution of the chemotherapy of more acute diseases, permitting greater concentration on some of the more difficult diseases such as tuberculosis and brucellosis. In this renewed interest, however, it must not be forgotten that the chemotherapy of tuberculosis has been under study for many years, and the numbers of compounds which have failed to show therapeutic value are great. Three years ago, D'Arcy Hart[1] reviewed the research during the past 100 years in this field. This interesting review reminds one that the specific questions still unanswered today were being asked at least 60 years ago.

Although a moderate degree of success has been possible in attempting to devise a rational approach to the improvement of therapeutic action of synthetic compounds in certain chemical series and against some infectious agents causing acute infections, in general, the approach remains disappointingly empirical, especially so in tuberculosis. Here, one wonders whether Work and Work,[2] in their book on chemotherapy, are not perhaps too optimistic for the present in saying: "In chemotherapy the transition state has been reached; theory has overtaken fact and has begun to shape the future." These authors, in attempting to analyze the relationship of chemical structure to chemotherapeutic activity, pointed out that in studying the effect of change in structure, several considerations should be kept in mind: "(1) Factors affecting distribution of drugs in animals. (2) Factors affecting the *in vitro* action of drugs and penetration of the bacterial cell wall. (3) Factors affecting the interaction of drug with enzymes."

When a specific series of related compounds are under study, it is sometimes possible to explain the behavior of one member of the series on the basis of information obtained on another, but, when large numbers of unrelated compounds are under study, generalizations are almost impossible to make. Yet, efforts in this direction must be made if an escape from the purely empirical approach to chemotherapy is ever to be attempted. In the present paper, very preliminary, perhaps even rudimentary, efforts will be described.

It will be recalled that White, Bratton, Litchfield, and Marshall,[3] after studying 126 compounds, many of which were sulfonamides, *in vitro* against a strain of type I pneumococcus, a strain of *Streptococcus viridans*, and a strain of β-hemolytic *Streptococcus*, as well as *in vivo* against the latter organism in experimental infections in mice, came to the following conclusions: (1) No compound is active *in vivo* if it is inactive *in vitro* on *Strepto-*

coccus unless it is decomposed in the animal organism to a compound which would be active *in vitro*. (2) Some compounds are active *in vitro* but inactive *in vivo*

Youmans, Feldman, and Doub[4] came to similar conclusions after making *in vitro* and *in vivo* comparisons of 33 derivatives of N,N′, diaminodiphenyl sulfone, using *Mycobacterium tuberculosis* as the test organism. In fact, these authors found agreement between *in vivo* and *in vitro* results with 22 out of 33 compounds tested. That is, both tests were positive or negative for the same compound. Eight of the 11 remaining were active *in vitro* but inactive *in vivo*.

Findings such as the above probably led Work and Work[2] to state: "A general conclusion which can be drawn from all recent work on bacteriostatic and bactericidal action *in vitro* is that no substance which is active *in vivo* is inactive *in vitro*. To this must be added the qualification that a compound which is inactive *in vitro* may appear to be active *in vivo* because in the animal it is transformed into an active form." In the next paragraph, these authors hasten to point out what many of us have found too often to be the truth, that is, that innumerable compounds are inhibitory *in vitro* but are ineffective in the body. It is specifically the latter problem which led the present authors to further attempts to correlate *in vitro* and *in vivo* activity in tuberculosis.

It should be kept in mind that, for many decades, investigators have found compounds active *in vitro* which failed *in vivo*. Koch probably suffered one of the earliest disappointments of this sort when he found that mercuric chloride, which had great activity against the anthrax bacillus in the test tube, failed to affect the disease in animals. Such failures are probably closer to the rule than the exception, especially in tuberculosis. Of more than 3500 compounds studied in our laboratories, approximately 40 per cent were inhibitory for BCG in a carefully standardized *in vitro* test, whereas only a small fraction of this number were significantly active in our standard *in vivo* tuberculosis test in the mouse using *M. tuberculosis* (Ravenel).[5] When references to a test of any compound *in vivo* against tuberculosis are made in the remainder of this paper, it will be understood, unless otherwise stated, that references are made to this standardized test. That BCG gives a good measure of *in vitro* action of compounds against virulent strains of *M. tuberculosis* was established early in the work by testing many compounds against the Ravenel and H-37Rv strains as well as BCG. Very good correlation was found to exist here.

Since many compounds are active *in vitro* but few are active *in vivo* in this disease, the question arises as to the significance of the *in vitro* test. Does it expedite the search for compounds active in the body? If we accept the thesis of Bratton, *et al.*,[3] that only compounds active *in vitro* (or potentially active *in vitro*) can be active *in vivo*, then we at least have made somewhat of a gain, by eliminating approximately 60 per cent of the compounds from further consideration because of lack of activity *in vitro*. This, however, still leaves many compounds having a minimal inhibiting concentration (M.I.C.) for BCG of 0.1 mg./ml. or less.[*] What other steps may be taken to reduce the size of this group of compounds to a workable number so as to permit a rational selection of compounds for more thoroughgoing studies?

As mentioned earlier, Work and Work[3] noted that it was important to keep in mind the factors affecting drug distribution in the body, factors affecting *in vitro* action of the drugs, and factors affecting the interaction of drugs and enzymes. Certainly these are worthy aims but most difficult of attainment when dealing with new types of compounds whose mode of action and behavior in the body are unknown. Complete studies of this nature are difficult and extremely time-consuming and, at best, progress would be very slow. If possible, it would be most helpful to ascertain the minimal limits of information required to explain the action of each new type of compound. Perhaps something may be gained by first considering what is already known about several types.

Quaternary Ammonium Salts. In general, these compounds are highly active against BCG *in vitro*, but none of them has as yet been found to be active *in vivo*. Ready adsorption by plasma proteins, with consequent inactivation in the body, appears to explain this phenomenon. Hence, in our work, these compounds have not been given serious consideration.

Heavy Metal Salts. Here again, although *in vitro* activity may be great, failure to show therapeutic action in the body is the rule. Besides problems of adsorption, toxicity is a common factor in this group, with a lack of selective action between host cells and bacterial cells.

Sulfonamides. Although, in experimental tuberculosis in guinea pigs[6, 7] and in mice, some of these compounds have shown a low order of activity, many have proven disappointing. The reason for this is not understood. There may be a clue to this failure in the manner in which these compounds behave *in vitro* versus BCG. We have noted, in this group, that, almost invariably, partial inhibition of growth may be caused *in vitro* at concentrations as low as 3 μg./ml., but complete inhibition is not caused by even thirty times this concentration. A study of the growth curves of BCG in the presence of such concentrations of the sulfonamides indicates that growth proceeds slowly but steadily.

Sulfones. A number of sulfones, most of them derivatives of N,N′,diaminodiphenylsulfone, have been found to be active in experimental tuberculosis in animals as well as moderately active in man. None, however, appears to be more active than the somewhat toxic parent compound (diaminodiphenylsulfone) itself. Although this cannot as yet be considered to be a closed field, it appears to be limited. There is good evidence to show that N-substituted derivatives of diaminodiphenylsulfone are active to the extent that they are converted in the body to the parent compound. No heterocyclic sulfone synthesized thus far has proven to be more active than the diaminodiphenyl compound.

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[*] In this field, the separation between so-called "active" and "inactive" compounds has commonly been on the basis of demonstrated inhibitory action, or lack of it, of a compound in a concentration of 10 mg percent, or 0.1 mg./ml. In our work, we have found that this is not an especially high order of magnitude of activity against BCG, with hundreds of compounds being inhibitory at 1/100th this concentration.

Thiosemicarbazones. This is a relatively new field and is being actively pursued both in this country and in Europe. A number of these compounds appear not only to be active *in vitro* and in experimental tuberculosis in animals,[8, 9] but also to be promising in man where final evaluation remains to be completed.

Of the groups of compounds which have given promise of activity against tuberculosis *in vivo*, all have been active *in vitro*. This includes not only the sulfonamides, sulfones, and thiosemicarbazones discussed above, but also streptomycin and *p*-aminosalicylic acid, although the latter appears to be required in higher doses than might be anticipated from its *in vitro* activity. Is there some characteristic about these compounds which distinguishes them from the many types of compounds which, though active *in vitro*, fail in the body? Preliminary attempts to find such characteristics are to be described in this paper.

It was decided early in our work that, ideally, an antitubercular compound should be active when administered *per os*, since it was anticipated that long periods of treatment would probably be necessary. This, of course, added still another handicap in finding new leads in this field of chemotherapy, but this was done deliberately, since it was considered unlikely that it would be readily possible to find a synthetic compound more effective than the streptomycins administered by a parenteral route.

Since our *in vitro* screen was turning up great numbers of compounds active in this test, the problem of selection of compounds for *in vivo* test was, and remains, a real one. Attempting to select a type species for each of the chemical series under study is very treacherous, since one could only guess what effect structural changes had on absorption and distribution. It was soon evident that selection on the basis of *in vitro* action alone, even in a given series, was inadequate. As data on many types of compounds accumulated, efforts were made to seek other criteria, and it is these efforts which we wish to discuss here.

When a compound is highly active *in vitro* and inactive *in vivo*, one may first seek to determine the degree of absorption of drug from the intestinal tract. However, there is often no known method of chemical analysis for new types of compounds. To attempt to devise an analytical method for each new type is an almost hopeless task. Biological assays of serum may be carried out, but the results are far from illuminating. First, the occurrence of active compounds in the blood stream is by no means an indication that the compound administered is the one showing activity. Furthermore, an even more disturbing occurrence is observed with some of the thiosemicarbazones which are active *in vivo*. The serum of mice kept on a drug diet at a level of proven therapeutic value shows little or no inhibitory action *in vitro*.

It is obvious, therefore, that demonstrating activity of a compound *in vitro* is a very far cry from finding an effective therapeutic. It is also obvious that a tremendous pharmacological step intervenes between these two stages, and it is because of the difficulty of making this second step that short cuts between the two must be sought. For this purpose, we must make certain assumptions. Let us assume that the M.I.C. of any given compound *in vitro* is also the M.I.C. which must be reached in the blood stream to obtain minimal therapeutic effects. The question then arises: "What dose of drug in the diet will give this concentration of drug in the blood stream?" For many compounds, there will be no assurance that ready absorption occurs from the intestinal tract and there may be no easily available chemical method for determining this. Therefore, for the moment, let us make the further assumption: that the new compound is absorbed and metabolized in a manner similar to that of some known compound. For this model compound, diaminodiphenylsulfone may be a good example.

In dogs, at least, this compound is entirely absorbed from the G.I. tract, as can be demonstrated by comparison of blood levels and urine levels following a given dose either *per os* or intravenously. Furthermore, 50 per cent of the dose will apparently be metabolized, since only half of the dose given can be recovered in the urine. Experimentally in mice, we have established the relationship between drug intake and blood level for this drug. This is demonstrated in TABLE 1.

TABLE 1

DIAMINODIPHENYLSULFONE

1. Absorption from G I. tract is essentially complete.
2. Approximately 50% metabolic destruction of drug occurs
3. When administered in diet·

$$\frac{\text{Blood Level}}{\text{Daily Dose}} = \frac{3.9 \text{ mg./l.}}{26.8 \text{ mg./kg.}} = 0.14.$$

4. Let

$$R = \frac{\text{Expected Blood Level}}{\text{Blood Level Required for Activity}} = \frac{\text{Daily Dose} \times 0.14}{\text{M.I.C.}}.$$

It can be seen that, when the daily drug intake in the diet was 26.8 mg./kg., the blood level, determined chemically, was 3.9 mg./liter, giving a blood-level/drug-intake ratio of 0.14. Now, if we go back to our assumption that, if the drug is to show therapeutic action *in vivo*, this blood level must be at least as high as the M.I.C. for the drug *in vitro*, we may relate the drug intake to the number of minimal inhibiting doses of drug theoretically present in the blood. This relationship we have termed "R." If there is any value to the assumptions which have been made, then the drug should be active *in vivo* when $R = 1$, or greater, and inactive when R is significantly less than one. In other words, unless the drug is tolerated in the diet at a level which will give the theoretical blood concentration required, it can scarcely be expected to be active *in vivo* unless the activity in the blood is greater than that *in vitro*. Now let us see how these calculations work out for several drugs of known activity in experimental tuberculosis in the mouse. Such data are shown in TABLE 2.

It can be seen that this approach works excellently in the case of diaminodiphenylsulfone and with *p*-formylacetanilide, 3-thiosemicarbazone.

In the former, a trace of activity is noted where R is 0.7, and the activity is definite and reproducible when R is greater than 1. Similarly, in the latter, evidences of some activity are noted when R is 1, and it is definite and reproducible when R is greater than 1. On the other hand, in the case of *p*-aminosalicylic acid, R must be somewhere between 33 and 98 before activity is noted. In none of these cases does serum seriously interfere with the *in vitro* activity, at least as measured by the use of 5 per cent sheep serum. Since *p*-aminosalicylic acid is rapidly absorbed from the G.I. tract, the interesting question remains as to why so much more of this compound is required than might be expected from the *in vitro* tests. Since the scope of the present paper is being limited to general considerations on many types of compounds, detailed discussion of this question will not be attempted at this time. Two points will be made in passing: (1) PAS, though showing partial inhibition of growth of BCG *in vitro* in our tests at a concentration as low as 0.8 μg./ml., does not cause complete inhibition at even several hundred times this concentration; (2) PAS is rapidly excreted from the blood stream. Both of these factors may contribute to the relatively large drug intake required for *in vivo* activity.

TABLE 2
"R" VALUES OF SEVERAL DRUGS

Drug	*Drug intake mgs./kg /day*	*R*	*Activity* in vivo
Diaminodiphenylsulfone	146	2.5	+
	66	1.1	+
	39	0.7	±
	17	0.3	−
p-aminosalicylic acid	1600	200	+
	785	98	+
	260	33	−
p-formylacetanilide-3-thiosemicarbazone	65.6	16.4	+
	36	9	+
	15	3.7	+
	4	1	±
	2	0.5	−

Similar calculations of R values for certain other well-known drugs, such as sulfathiazole and some other thiosemicarbazones, yield results much like those with diaminodiphenylsulfone, *i.e.*, when R is 1 or greater, activity is noted *in vivo* and, when it is less than 1, no activity is found. It appeared worth while therefore to compare R values and *in vivo* activity for a large number of heterogeneous compounds which we have tested both *in vitro* and *in vivo*. Such data for 379 compounds are summarized in TABLE 3.

In this table, it will be noted that the number of compounds showing no activity at the maximal acceptable drug level in the diet, the number showing traces of activity at this drug level, and the number showing definite activity at each R value level are given. Where definite activity was found, only the lowest R value at which it was obtained is listed in this summary. It is obvious at first glance that the distribution of compounds according to R values does not make any separation between active and inactive compounds. In other words, there is just as high a proportion of drugs showing no activity at high R values as there is at low R values. and, similarly, there is just as high a proportion of drugs showing activity at low R values as there is in the high R value range. This, of course, is disappointing, but the authors realize that this is only a first step in attempting to rationalize the relationship between *in vitro* and *in vivo* results. What further steps can be taken?

If we go back to TABLE 1, it can be seen that, at a given daily drug intake, the R value decreases as the M.I.C. increases. Therefore, if interfering substances occur in the blood stream, the effective M.I.C. will rise, and the R value will drop. Hence, our next efforts were devoted to testing the effect of serum on many of these compounds.

TABLE 3
RELATIONSHIP BETWEEN "R" VALUES AND *in vivo* ACTIVITY IN EXPERIMENTAL TUBERCULOSIS

R	*Degree of activity*						*Totals*	
	−		±		+			
	No.	*%*	*No.*	*%*	*No.*	*%*	*No.*	*%*
<1	78	74.3	11	10.5	16	15.2	105	100
1	40	75.5	5	9.4	8	15.1	53	100
2	43	76.8	4	7.1	9	16.1	56	100
4	38	70.5	5	9.3	11	20.2	54	100
8	27	67.5	3	7.5	10	25.0	40	100
16	22	78.6	3	10.7	3	10.7	28	100
32	15	68.2	3	13.6	4	18.2	22	100
64 or greater	15	71.5	3	14.3	3	14.3	21	100

In our tests, it was found that the rate of growth of BCG *in vitro* was reduced by high concentrations of serum. After testing the serum of several species of animals, it was decided that sheep serum caused the least amount of retardation and that as long as no more than 5 per cent sheep serum was added, normal growth occurred. Hence, in the tests on the effects of serum, 5 per cent sheep serum was used. Seventy-five compounds were tested in this manner. These findings are listed in TABLE 4.

The *in vitro* activity of 30 per cent of the compounds was not significantly affected by the presence of serum. In another 32 per cent of the compounds, the M.I.C.'s were increased 3- to 5-fold by this treatment, and, with still another 25 per cent of the compounds, this increase was from 6- to 10-fold. With approximately 11 per cent of the compounds, this increase was greater than 11-fold. It was, therefore, considered important to recalculate the R values for all these compounds, correcting the M.I.C. for the serum affect. When this had been done, the compounds were again listed according to +, ±, and − activity *in vivo*, and the relationship between R value and activity was re-examined. These results are listed in TABLE 5.

Even with the correction for serum taken into account, it can be seen that no separation of active and inactive compounds could be made on the basis of R values. It is therefore quite reasonable to ask whether anything has been gained by this type of analysis. As far as immediate clarification of the relationship between *in vitro* and *in vivo* results is concerned, one must confess that the answer is no; but it did yield something which the authors were seeking from the outset, that is, a means of separation of the compounds into groups permitting a selection of compounds for further study. Hence, those compounds which fail, but have R values of less than 1, were dropped from further consideration for the time being, because their toxicity or acceptability did not permit a sufficiently large dose to be given to attain an inhibitory blood level, even if one assumes that they were completely absorbed. This, in itself, eliminated a large number of compounds.* Further, those compounds which fail, although they gave very high R values

TABLE 4

EFFECT OF 5% SHEEP SERUM ON M.I.C. OF VARIOUS ANTITUBERCULAR COMPOUNDS

Increase of M I C. in presence of serum										*Totals*	
1 to 2 fold		*3 to 5 fold*		*6 to 10 fold*		*11 to 20 fold*		*> 21 fold*			
No of compounds	*%*	*No. of compounds*	*%*	*No. of compounds*	*%*	*No. of compounds*	*%*	*No of compounds*	*%*	*No of compounds*	*%*
23	30.7	24	32.0	19	25.4	5	6.7	4	5.3	75	100

TABLE 5

"R" VALUES CALCULATED USING SERUM EFFECT

R	− *Inactive*	±	+ *Active*
<1	6	1	1
1–2	2		4
2–4	4		1
4–8	7		2
8–16	2	1	1
16–32	6		1
32–64	1		0
>64	2		0
Total	30	2	10
		42	

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* Here it should be emphasized that our drug acceptability tests are by no means a certain test for toxicity The maximum drug level used in the *in vivo* tests was that level on which mice at least maintained their starting weight If a given level caused a weight loss it was tentatively considered toxic However, it is recognized that weight loss may have been due to refusal of the mice to eat the diet because of taste or other undesirable characteristics of the drug, and not necessarily due to toxicity Some compounds of high *in vitro* activity showing very low acceptability are now being re-examined to determine their true toxicities Should these prove to be significantly lower than that indicated by the acceptability tests, the drugs will be retested in infected mice by administering the drug by means other than in the diet.

in the presence of serum, appeared to be worthy of concentrated attention. The first consideration of course is whether these compounds are absorbed from the diet. Specific reversing agents may be sought for those which appear to be absorbed.

It is also felt that more work must be done on the effect of serum on many compounds. Since, in order to permit maximal growth of the test organism, we have been limited to the use of only 5 per cent serum, while in the body the drug is exposed to 100 per cent serum, the nature of the slope of reversal of various compounds by serum must be studied. In this way, one might be able to predict that the effective M.I.C.'s in the body are even lower than those used in our calculations thus far.

A further step in the process is to attempt to explain why some compounds are active even when the R value is significantly lower than 1. These are very few in number, but the implication here is important. It would seem that these compounds may be more active *in vivo* than *in vitro*. If this is the case, there is the possibility that they have been converted in the body to something even more active than what was administered (a compound such as Promin would fit into this category) or that the body provides some factor which enhances the activity of the given compound. Studies along these lines are proceeding at present in our laboratories.

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EVALUATION OF THE PAPER DISC ASSAY PROCEDURE AND ITS CORRELATION WITH PHENOL COEFFICIENTS

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Historically, the F.D.A. phenol coefficient test has had the widest application of any test procedure in the evaluation of disinfectants and antiseptics, in spite of the abuse heaped upon it. The variables which must be rigidly controlled if this test is to be reproducible and the limitations of interpretation which must be exercised, lest one place too much emphasis on the value of a disinfectant based upon its phenol coefficient alone, have been the subject of so much discussion in the past that it is hardly necessary to refer to them here. However desirable it might be, it appears certain that no one simple test will ever give a complete picture on any particular disinfectant or antiseptic. Yet, in spite of this, laboratories continue to need, and to use, some such easily conducted test, if only to gain a preliminary evaluation of the performance of a new disinfectant.

In this laboratory, in connection with a program in which we are continually searching for new and better disinfectants, several simple screening tests are routinely employed on new compounds as they become available. The paper disc assay technique, largely developed by those interested in antibiotics, has appealed to us as informative, simple to conduct, and applicable to the evaluation of various groups of antiseptics as well as the antibiotics.

The work reported upon here is concerned with the evaluation of a group of phenolic compounds utilizing this paper disc assay and with an attempt to correlate these data with phenol coefficients on the same compounds.

In order to apply this procedure to the evaluation of phenolic compounds, it was necessary to determine exactly what variable factors had to be rigidly controlled if the zones of inhibition obtained were to be reproducible.

These variables will be stated with no supporting data. First, the agar in the plates must be standard. This means not only using agar of a definite composition and pH but having a fixed amount of agar in each dish and selecting Petri dishes that are flat-bottomed. These latter two factors control the depth of agar underneath the paper discs. The shallower the agar under the disc, the wider the zone of inhibition will be. Second, the inoculum must be constant. Not only do different organisms vary greatly in resistance, but various strains of the same organism can give different results. The age of the culture used to seed the plate must always be the same, as well as the manner in which it is grown. The amount of inoculum should be always the same, or, more exactly, the number of organisms per unit area of agar should be as constant as possible. The time elapsing between the seeding of the plates and the placing of the paper discs on the surface of the agar should also be standardized. Third, the amount of chemical added must be constant. This means that, in addition to having the concentration of the chemical fixed, a standard volume of this chemical must be added to a paper disc of standard size and weight. Fourth, the incubation after adding the chemical must be constant, as to both time and temperature.

None of these fixed conditions of testing is in any sense peculiar to the use of the paper disc assay for the evaluation of phenolic or other compounds. They have been discussed by the various authors publishing on the use of this technique in evaluating antibiotics (Schmidt & Moyer, 1944). The testing technique adopted in this laboratory is as follows:

A nutrient agar is used which contains 0.3 per cent beef extract, 1.0 per cent Bacto peptone, 0.5 per cent yeast extract, 1.0 per cent dextrose, and 2.0 per cent Bacto agar. Fifteen ml. of this melted nutrient agar are pipetted into flat-bottomed petri dishes and allowed to harden. The plates are then seeded with 5 ml. of inoculated agar, made by adding 0.3 ml. of a 24-hour broth culture of *Micrococcus pyogenes*, var. *aureus* (*Staph. aureus*, 209 strain), to 180 ml. of the same nutrient agar described above. Immediately after the seeded agar has hardened, a standard penicillin assay disc (Vincent & Vincent, 1944) is placed in the center of the plate. The antiseptic being tested is made up in the desired dilutions in distilled water, or in alcohol if insoluble in water,* and 0.08 ml. of the solution is pipetted onto the paper disc. This seems to be the greatest amount that the paper can absorb readily. The plates are then incubated at 37° C. for 16 to 18 hours, while placed upright in the incubator with clay tops used to prevent moisture collection. The diameter of the zone of inhibition is then read to the nearest half millimeter. Since the disc itself has a diameter of 12.7 mm., this is the minimum figure for a zone of inhibition.

The width of the zone varies with the concentration of disinfectant in a logarithmic fashion, as is shown in FIGURE 1. Here the data for phenol itself and seven substituted phenolic compounds are given. By using semilog paper and plotting concentration of disinfectant against the zone of inhibition, good straight lines can be drawn through the points for each disinfectant. The dotted line drawn in at a diameter of 12.7 represents the size of the paper disc, and hence indicates the concentration below which no zone of inhibition is observed. The slopes of the lines, it will be noted, do vary. This may be due to differences in the rates of diffusion of the various compounds, but possibly this is not the only factor involved. Among the compounds shown here, the quite insoluble and heavy pentachlorophenol has the steepest slope. Only tri-iodophenol, not reported here because its phenol coefficient could not be readily determined due to its insolubility, showed a steeper slope. The steepness of the slope does not necessarily follow solubility and molecular weight relationships, since, as can be readily seen, phenol itself, with the greatest solubility and lowest molecular weight of the group, also has a greater than average slope.†

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* Alcoholic solutions of phenol and aqueous solutions of the same strength give identical zones of inhibition. Alcohol itself does not produce a zone of inhibition. The data reported here are all with alcoholic solutions, since so many of the compounds are so slightly water soluble. This ability to separate the effect of the disinfectant from that of alcohol in tinctures is one of the advantages of this testing technique.

† The analogy between the diffusion of various compounds through agar and the results obtained in paper chromatography was brought out in the discussion following this paper. In chromatography, also, it is difficult to correlate the diffusion obtained with the physical properties of the compounds under test.

One might expect at first that extrapolation back to the zero zone of inhibition would indicate the concentration of chemical in agar which is just inhibitory to the growth of *Staph. aureus*. The minimum concentration of phenol which, when dissolved in agar, will inhibit the growth of *Staph.*

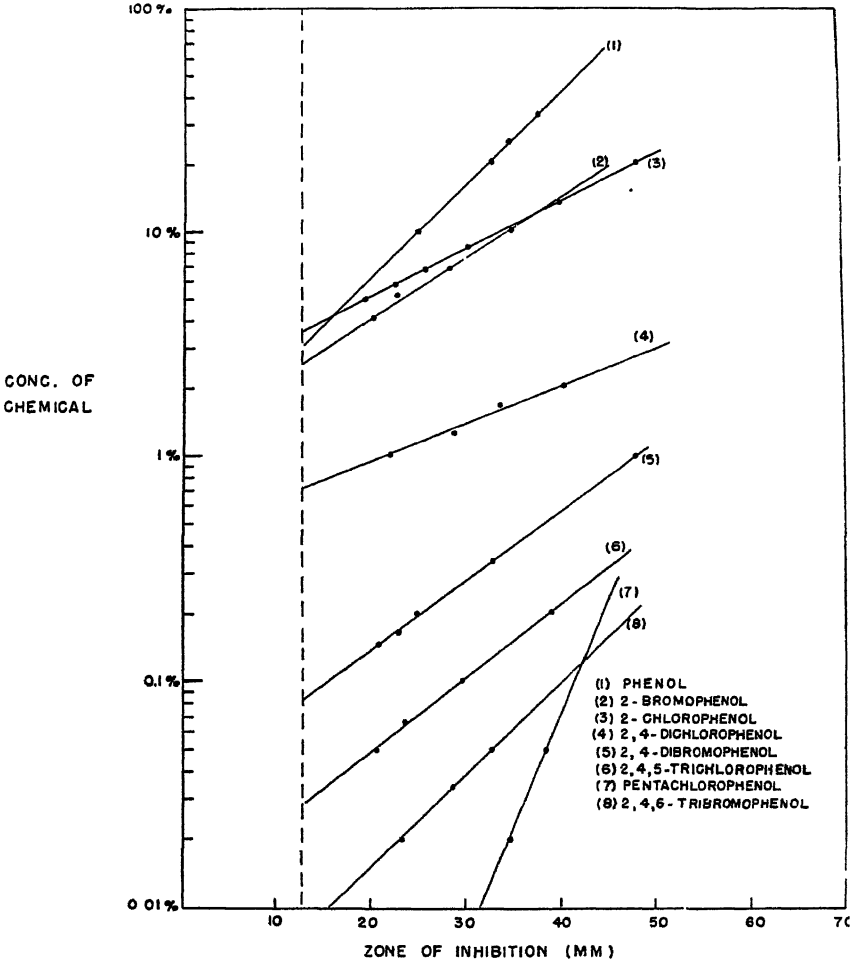

FIGURE 1. Paper disc assay zones of inhibition for various phenols.

aureus is between 0.3 and 0.5 per cent. Interpolating the phenol line on this graph back to the 12.7 mm. or zero inhibition zone gives a minimum inhibitory value of 3.2 per cent. If the amount of agar in the plates is reduced, however, the zones become wider, and the extrapolated concentration which gives no zone of inhibition becomes lower. With only 5, rather than 20, ml. of agar in the plate, this figure drops from 3.2 to 1.3 per cent. If excessively thin plates could be poured and read, perhaps the figure might approach the 0.5 per cent minimum inhibitory value for phenol dissolved in solid media.

Our main interest in these lines, however, is to compare the relative efficiency of these different compounds. To do this, an arbitrary value, the concentration required for each compound to produce a 30 mm. zone of inhibition, was read from the graph in FIGURE 1. Each such concentration, in turn, was then divided into the concentration of phenol required to produce this same effect, and these values were called 30 mm. zone ratios. If the slopes were all the same, the ratios would be identical for any particular zone of inhibition which might have been selected. Since the slopes vary, however, the choice of which zone to use as a basis of comparison must be arbitrary, at least until the significance of the differing slopes is better understood.

TABLE 1
COMPARISON OF RATIOS WITH PAPER DISC ASSAY AND PHENOL COEFFICIENTS

Chemical	*30 mm. Zone ratio*	*Phenol coefficient*
Phenol	1.0	1.0
2-bromophenol	1.83	3.7
2-chlorophenol	1.87	3.8
Benzoic acid	2.82	6.25
3-chlorophenol	4.5	5.8
2-hydroxy benzoic acid	5.63	10.0
2,4-dichlorophenol	11.6	12.7
2,4-dibromophenol	52.2	22.0
2,4,5-trichlorophenol	154.0	27.0
2,4,6-tribromophenol	364.0	33.0*
Pentachlorophenol	1927.0	50.0*

* Performed in this laboratory. The other values for phenol coefficients are those found in the literature.

In TABLE 1 are shown the values obtained for these 30 mm. zone ratios. The published phenol coefficients for these same compounds appear in the second column. For the last two compounds, which are starred, no recorded phenol coefficients could be found; these were therefore run in this laboratory. With the simpler compounds, the zone ratios are somewhat lower than phenol coefficients, but, with the more active compounds, the results were startling. 2,4,5-Trichlorophenol, which had a phenol coefficient of 27.0, produced a 30 mm. zone at 1/154th of the concentration required for phenol. 2,4,6-Tribromophenol was 364 times as active as phenol, and pentachlorophenol showed up at almost 2,000 times as active.

In FIGURE 2, one will observe what happens when the logarithms of these 30 mm. ratios, rather than the ratios themselves, are plotted against phenol coefficients for the same compounds. A good straight line can be drawn through these points, with the exception of the value for pentachlorophenol. The phenol coefficient for that compound is not too certain, since alcohol must be used to get enough of the compound into solution for testing; but, as will be brought out later, it is not too unexpected that the relationship begins to break at this point.

Unlike Tobie and Ayres (1944), who ran comparative tests on quaternary compounds, using these same two techniques, and could find no correlation, we find that a relationship between the tests does appear to exist, at least with the closely related phenolic compounds reported here Not only do the compounds line up in the same order of relative strength in the two tests, but a definite mathematical relationship appears to result. Why this relationship should be exponential is somewhat obscure. It is merely desired to point out here that it exists and that it seems to hold true with this group of compounds, up to those having a phenol coefficient of around 35, where the 30 mm. zone ratio is about 500. More data are needed to see how much further this relationship holds. A break appears to occur with pentachlorophenol, but more work should be done with compounds having very high phenol coefficients, such as with some of the alkyl chlorophenols investigated by Klarmann *et al.* (1933). If this logarithmic ratio should remain constant over a wide range, some of the compounds reported as having a phenol coefficient around 500, for example, would have to show a 30 mm. zone ratio to phenol of about 10 to the 40th power, a truly astronomical figure. Needless to say, we do not expect to find any such values. It would also be interesting to see if this logarithmic relationship between zone ratios and phenol coefficients exists when other groups of disinfectants are tested, aside from the phenolic compounds reported upon here and the quaternary compounds for which Tobie and Ayres (1944) failed to find any relationship.

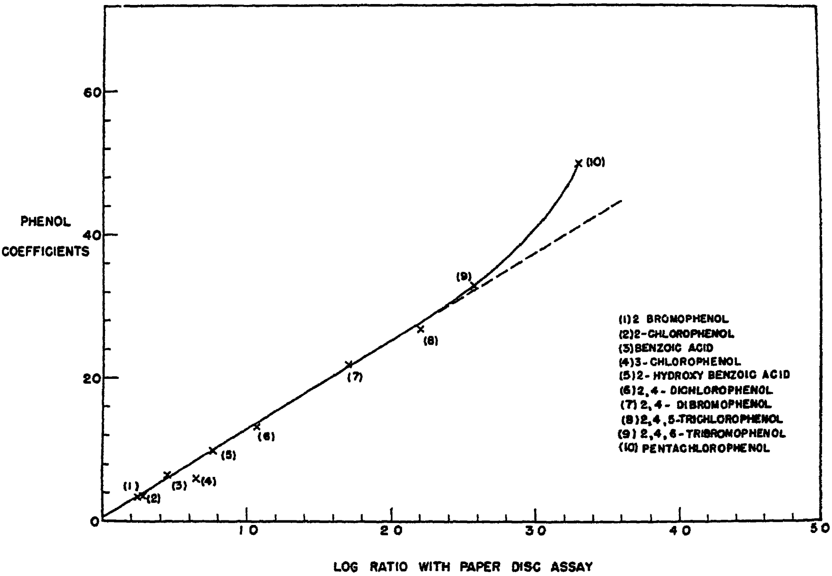

Figure 2. Comparison of ratio with paper disc assay and phenol coefficients

The paper disc assay can also be easily adapted to show the selectivity of antiseptics towards various groups of bacteria, by using a spectrum of test organisms. FIGURE 3, for example, shows how two closely related chlorophenols react towards a spectrum of organisms. 2,4,5-Trichlorophenol exhibits similar activity towards the five organisms, *Serratia marcescens*, *Eberthella typhosa*, *Escherichia coli*, *Bacillus globigii*, and *Staphylococcus aureus*. Pentachlorophenol, on the other hand, is much better against the Gram-positives in this group than against the Gram-negatives.

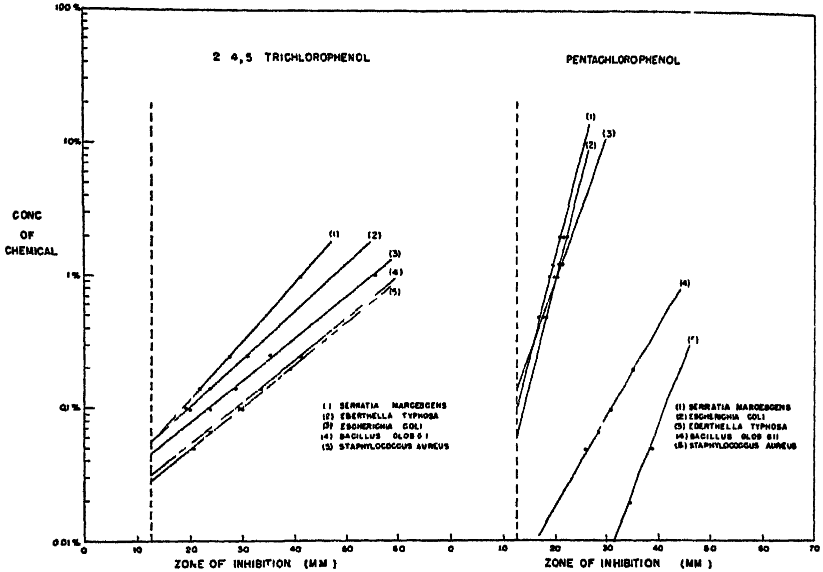

FIGURE 3. Paper disc assay with various test organisms.

TABLE 2

SELECTIVE ACTION OF VARIOUS PHENOLIC COMPOUNDS

Compounds having universal action	*Compounds selective for Gram + organisms*
Phenol	Pentachlorophenol
2,4-dichlorophenol	2,6-dinitro-4-octyl phenol
2,4,5-trichlorophenol	4,6-dinitrophenol
2,4,6-trichlorophenol	2-isopropyl phenol
	2,6-dinitro-4-chlorophenol
	4,6-dinitro-2-methyl phenol

This phenomenon has been observed before in certain substituted phenols. Suter (1941), in summarizing the existing bacteriological information on the phenols, lists many cases where compounds were tried against more than one test organism (usually phenol coefficients with *Staph. aureus* as one test organism and *E. coli* or *E. typhosa* as a second organism). In almost all of the cases reviewed by him, where a difference was noted, the phenolic compound in question was more active against the Gram-positive than the Gram-negative organism. A notable exception was hydroquinone, whose phenol coefficient with *E. typhosa* was about 25 times higher than its coefficient with *Staph. aureus*. Klarmann (1933), who ran phenol coefficients with as many as six test organisms, found with certain compounds a much higher phenol coefficient with certain of the Gram-positives and called this a "quasi-specific effect."

In TABLE 2, we list certain of the phenolic compounds, including phenol itself, for which we found no selective action towards the five test organisms used, and certain typical substituted phenols in which the selective action for Gram-positive organisms is pronounced.

The data presented here have been given to show some of the uses to which the paper disc test has been placed in this laboratory. In the study on phenols, we have been particularly interested in finding an apparent correlation between this test and phenol coefficients, and in having a method whereby compounds soluble only in alcohol could be tested. True, the paper disc test measures bacteriostatic rather than bactericidal action. The F.D.A. test, also, is primarily a bacteriostatic one, depending upon dilution and subculturing for neutralizing the disinfectant being tested, except where a definite neutralizer is used, as in modifications calling for thioglycollate when testing mercurials. For preliminary evaluation, bacteriostatic tests are not particularly disadvantageous, since a new compound must at least show this activity before it warrants further study. In both tests, large amounts of organic material are present, and, in the paper disc test, the compound must diffuse through agar. This could possibly mask the effectiveness of certain groups of disinfectants, as, for example, the quaternaries (M. A. Phillips, 1947). The paper disc assay is not suitable for testing volatile disinfectants such as alcohol, but this factor, as mentioned above, can be of definite use. The technique certainly has definite advantages in the simplicity and speed with which tests can be run.

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MECHANISM OF ACTION AND NEUTRALIZING AGENTS FOR SURFACE-ACTIVE MATERIALS UPON MICROORGANISMS

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In this paper, it would seem desirable to orient the informed, as well as the uninformed, workers in the fields of disinfection and antisepsis as to some of the theories that have been advanced concerning the possible mechanisms of action of "surface-active agents" upon microorganisms. Since several of the papers presented in this monograph include a discussion of "neutralizers," "inactivators," or "antidotal" substances which may be used to counteract the antibacterial activity of surface-active germicides, brief mention will also be made of this subject.

Some of the compounds that were incorporated on the nucleus of hexamethylenetetramine by Jacobs and Heidelberger[1–8] may be considered surface-active agents. In one of the early publications by Jacobs,[9] certain conclusions were drawn in respect to the correlation between chemical configuration of the hexamethylenetetramines and their degree and specificity of activity against bacteria. Note was made of the fact that the bactericidal nature of the compounds was primarily concerned with the hexamethylenetetramine portion of the molecule, whereas the degree of activity was determined by the nature of the molecular groups attached to the latter. Jacobs also demonstrated that the added groups on hexamethylenetetramine were responsible for the derivatives, showing, in some instances, incompatibility with serum and the toxicity of others.

In studying the mechanism of action of a homologous series of alcohols of the primary, secondary, and tertiary types, Cowles[10] found that, against *Eberthella typhosa* and *Staphylococcus aureus*, the germicidal and surface activities increased with an increase in chain lengths of the alcohols. There appeared to be a close correlation between the germicidal action and surface activity of the alcohols, which suggested to him that these organic compounds may depend upon adsorption primarily and upon toxicity to bacterial protoplasm secondarily.

In studying the "detoxifying" action of various agents against bacteria, Harris and Bunker[11] found that tetramethylammonium chloride would destroy the virulence of bacteria under certain conditions without necessarily killing them. Inhibition of certain physiological processes of the bacterial cells were brought about by the germicide; yet the production of antibodies in the host continued. This action, observed with the tetanus organism, was attributed not to the surface-tension depressant action of the quaternary ammonium compound, nor to the particular anion or cation, but to the configuration of the molecule as a whole.

Neter[12] was able to demonstrate that amounts of as little as 0.1 mg. of a quaternary ammonium salt would detoxify 10 minimum lethal doses of tetanus toxin. This action was independent of any changes that could be attributed to the influence of pH. Serum would inhibit this detoxifying action No mention was made in the report as to whether or not the surface-active compound affected the antigenic properties of the detoxified toxin. Neter[13] also presented the results of his observations on the effect of a surface-active cationic germicide upon plasma coagulation by staphylococci and upon fibrinolysis by streptococci. The compound in relatively high dilutions delayed the clotting of oxalated human plasma by staphylococcus cultures and inhibited fibrinolysis by hemolytic streptococci.

Holmes[11] noted that the antigen in serological systems was not affected by an anionic detergent and that no change could be evidenced on bacteria, since the latter could still absorb antibody after treatment The same agent also failed to denature antiserum in the concentrations tested. While alcohols were found not to alter the course of the reactions in precipitation and agglutination tests, the soaps used in the investigation were found to inhibit immunological reactions. This interference was attributed to an increase in the electrical charge on the organisms by the anionic soaps. Cationic detergents, on the other hand, acted in an opposite manner by spontaneously agglutinating the organisms due to markedly lowering their charge. Thus, the latter compounds decreased the stability of the antigen and also caused a nonspecific precipitation of serum.

Miller, Baker, and Harrison[15] showed that a surface-active quaternary ammonium germicide would exert a pronounced inhibitory effect on respiration and glycolysis of organisms associated with dental caries. Concentrations as low as M/10,000 of the compound were effective in a matter of a few minutes. This action was complete and irreversible, inasmuch as the activity was not regained by washing the treated cells and again testing them for metabolic activity in fresh medium. In continuing these investigations, Miller and his co-workers[16–18] observed that the quaternary ammonium germicides (cationics) would inhibit the metabolism of Gram-positive and Gram-negative bacteria in concentrations ranging from 1/3000 to 1/60,000. The anionic surface-active compounds, on the other hand, showed complete inhibition only against the Gram-positive bacteria and here at a dilution of 1/3000. These workers implied that the complete absence of bactericidal action of anionic detergents and diminished activity of some of the cationic detergents against Gram-negative organisms may be related to differences in content, type, or cellular arrangement of phospholipids in the Gram-negative as compared to the Gram-positive bacteria.

Partial substantiation of the point just mentioned may be found in the report by Baker, Harrison, and Miller,[16] in which an investigation was made of the interfering action of phospholipids upon the antibacterial activity of a number of synthetic detergents. They had previously found that a combination of an anionic detergent with a cationic detergent (quaternary ammonium compound) would often result in the formation of a precipitate. This reaction was accompanied by an interference of the inhibiting action on bacterial metabolism by the anionic or cationic detergent. However, when phospholipids were added to a cationic detergent before or simultaneously with the addition of the latter to a bacterial suspension, although interference in metabolism of the cells would also occur, the reaction was considered quite different from that observed when cationic and anionic detergents were combined.

Using the manometric technique described by Baker and his co-workers, Ordal and Borg[19] studied the oxidation of lactate by molecular oxygen as compared with the anaerobic oxidation of methylene blue in testing the activities of an anionic and a cationic detergent against *S. aureus* and *Escherichia coli.* These investigators found that the lactate dehydrogenase of *S. aureus* is more susceptible to the action of both anionic and cationic surface-active agents than that of *E. coli.* While both types of detergents inhibited the oxidation of lactate by *S. aureus*, only the cationic detergent (quaternary ammonium compound) inhibited the oxidation of lactate by *E. coli.* With *S. aureus*, little difference was noted in the concentrations of both types of surface-active compounds required to inhibit the oxidation of lactate by oxygen, as compared to the oxidation by methylene blue.

Sevag and Ross[20] studied the mechanism of inhibitory action of a quaternary ammonium surface-active agent against yeast cells. A concentration of 1/1000 of the compound caused a marked fading or abolishment of the adsorption bands of a preparation of cytochrome C and yeast cells. In their manometric measurements, a 1/35,000 dilution of the quaternary ammonium germicide caused complete inhibition of the oxygen consumption of *p*-phenylenediamine in yeast cells, and the aerobic and anaerobic oxidation of glucose were inhibited.

Knox and his co-workers[21] found that many of the cationic surface-active compounds which they tested in their metabolic studies would kill certain bacteria in concentrations that inhibited the metabolic reactions of the same organisms. They were able to demonstrate that cell-free bacterial enzymes can be inhibited by cationic detergent-protein ratios which are bactericidal for the intact cells. Furthermore, the killing and inhibition of bacterial growth are proportional to the detergent-bacteria ratio and not to the concentration of the detergent used. Their studies revealed, moreover, that certain enzymes may be specifically sensitive to the cationic quaternary ammonium germicides, which accounts for the metabolic inhibition, cell death, and permeability noted in bacteria treated with bactericidal amounts of the compounds.

Hotchkiss[22] observed that the enzyme proteins or other vital parts of the bacterial cell appear to have a greater sensitivity to surface-active agents than do proteins in general. He noted that when bacteria are exposed to lethal concentrations of these compounds a leakage of nitrogen and phosphorus out of the cell would occur. This action can be demonstrated with cationic quaternary ammonium germicides, anionic surface-active compounds, cresols, bile salts, tyrocidin, and, to some extent, with nonionic surface-active agents. Germicides such as hydrogen peroxide, potassium permanganate, formaldehyde, organic mercurials, acridines, and the halogens failed to cause a leakage of cellular elements from bacteria.

Gale and Taylor[23] confirmed, in part, the observations of Hotchkiss in that they were able to demonstrate that organisms exposed to tyrocidin showed a loss of internal free lysin and glutamic acid. A similar action was noted when a quaternary ammonium surface-active compound and an anionic detergent were used as the suspending media. No such effect was obtained with gramicidin, penicillin, sulfathiazole, or acriflavine.

The conductometric measurement procedure described by Eggenberger *et al.*[24] has been the most recent approach to an explanation for the possible mechanism of action of surface-active quaternary ammonium germicides upon bacteria. Their findings reveal that cells exposed to certain of these compounds will release a highly conducting material as a means of protection for the organism. The rate of excretion of the protective material was found to increase with increased concentration of the germicide, terminating in a literal outpouring of the substance as the point of rapid killing is reached. Autoclaved bacteria treated with the same germicides failed to show this effect. Electron microscope photographs failed to reveal that this outpouring of conducting material was due to the rupture of the cells of the organism, inasmuch as the cells killed by the germicides were still intact.

The appearance of vacuoles and the enlargement of organisms exposed to penicillin as noted by Gardner[25] may also be an effect of surface activity of the latter antibiotic. Hauser, Phillips, and Vavruch[26] and Hauser[27] were able to demonstrate that penicillin solutions resemble soapy water and possess surface-active properties. These investigators found that the antibiotic bears an electric charge similar to soaps and has a tendency to cling to surfaces with an opposite charge. Using ultraviolet light microscopy, they found that penicillin coats bacterial cells, as evidenced by the bacteria taking on the fluorescent properties of the antibiotic. The partial lysis of human tubercle bacilli in the presence of high concentrations of penicillin, as noted by Kirby and Dubos,[28] may also be a function of the surface activity of the antibiotic.

Among the various effects of a quaternary ammonium surface-active germicide and anionic surface-active agents upon *Trichomonas foetus* reported by Lawrence[29] was the gross shrinkage and complete fragmentation of the protozoal cells. In all the media in which cell fragmentation occurred, the surface tensions of the detergents were between 30 to 40 dynes per centimeter. Dissolution of motile trichomonads by quaternary ammonium germicides was also observed by MacDonald and Tatum,[30] who studied the effects on *Tr. hominis*, *Tr. vaginalis*, and *Tr. foetus*.

Autolysis of protozoan cells, probably not unlike the action of quaternary ammonium germicides upon trichomonads, has been observed by Anderson,[31] who exposed cultures of trypanosomes to the antibiotic, subtilin, which was also found to possess surface tension depressant activity. He also reported that cultures of trophozoites of *Endamoeba histolyticum* would "rupture" when exposed to the antibiotic.

An extensive series of investigations was carried out by Taft and Strandtmann,[32–34] in which the effects of a quaternary ammonium surface-active germicide were determined against a variety of invertebrates. Among their observations was the rupturing effect of the compound on paramecia, colpidium, hypotriches, and planaria. While these and other organisms reacted differently to the germicide, this was not considered to be a phylogenetic difference but one due to the protective covering of the animals.

The action of the surface-active compound was attributed, to a large extent, to its ability to lower surface tension and to its wetting properties.

Kuhn and Bielig[35] found that proteins such as gelatin and egg albumen are precipitated in a water solution by quaternary ammonium compounds, whereas the latter fails to precipitate globulin. This latter phenomenon is attributed to the isoelectric point values of the proteins. Since proteins are precipitated only in the form of their anions, the negative-charged proteid ions will react with the positively charged molecule of the cationic compounds. This may also explain part of the mechanism of the killing action of the quaternary ammonium germicides on bacteria. Thus, the reaction of this group of compounds could be with the anion of the cell protein and the complexes essential for life in the bacterial cell. A similar explanation has been offered by Pfankuch and Kausche[36] for the mechanism of action of quaternary ammonium surface-active germicides against plant viruses.

Anson[37] noted that synthetic detergents and bile salts would denaturate proteins such as hemoglobin and egg albumen at their isoelectric points and keep these treated proteins in solution. Furthermore, if an excess of anionic or cationic detergent is added to bacteria, there is denaturation of enzyme proteins, some of which may be necessary for the lysis of certain organisms such as the pneumococci.

In studying the virucidal activity of saponin, sodium desoxycholate, and an anionic detergent against viruses, Burnet and Lush[38] observed a close correlation between the concentrations of the agents capable of hemolyzing rabbit's red blood cells and the effect against herpes virus. Of the eleven viruses tested, only psittacosis virus was completely insusceptible to the several agents mentioned. They postulated from their findings that the inactivation within the virus cell is a function of some enzyme capable of disintegrating the surface of the virus particle in a manner analogous to that of the activity of the surface-active agents. A similar hypothesis was advanced by Smith[39] for the mechanism of action of bile salts against viruses. This investigator also considered that virus inactivation was due to the presence of an autolytic ferment, and the rapidity with which the susceptible viruses are completely inactivated by sodium desoxycholate compares with the phenomenon of the bile solubility of the pneumococcus. This suggests that the virus elementary bodies may actually be lysed by bile constituents. This should be readily revealed through electron microscopy.

Albert[40] attributed the action of cationic antiseptics to their basic ions, which react with the acidic ions of microorganisms. Thus, these compounds have a greater effect against those organisms that contain an excess of acidic groups (nucleus and enzyme systems) over basic radicals. This may explain the general trend of greater activity of the quaternary ammonium germicides against *S. aureus* and other Gram-positive bacteria that contain a high ratio of acidic over basic groups than against Gram-negative organisms where the ratio is reversed.

Valko and DuBois[41] found that an active quaternary ammonium compound may be markedly diminished in its antibacterial action by a less active quaternary ammonium salt, even when the compounds contain reasonably comparable constituents in the molecules. They attributed this interference as a reaction that occurs on the surface of the bacterial cell or within the cell itself. Thus, certain spaces on or in the cell will specifically select the harmless cations and protect the organism from the more toxic cations.

Dyar[12] also noted a reaction between bacterial cell constituents and a surface-active mordant, which he used prior to the addition of an aqueous solution of Congo red to the organisms. Following washing and counterstaining with methylene blue, the cell walls of yeast and bacteria stained red, with definite evidence of a red precipitate. While the cytoplasm of the cells appeared to be shrunken away from the cell wall, he did not consider this indicative that the quaternary ammonium germicide had not penetrated the cell membrane. This was supported by evidence presented to show that the cytoplasm of the vegetative cells in the surrounding medium stained black when treated with a quaternary ammonium salt and a fat-dye, Sudan black B.

Dyar and Ordal[43] and Kivela, Mallmann, and Churchill[44] studied the electrophoretic mobilities of bacteria when treated with surface-active detergents. The first investigators found that anionic compounds produced no change, a slight change, or marked change in the electrophoretic mobility of the test organisms. Thus, if a change occurs, the concentration of the anionic compound at which this first occurs may vary considerably. With the cationic compound, there was a general trend of a decrease in charge on the cells, reversal of charge, and, finally, stabilization of charge. Kivela and his associates observed that by merely washing vegetative cells and spores of bacteria, following treatment with a quaternary ammonium germicide, one can restore the normal electrical charge of the cells. While revival of the washed bacterial spores could also be demonstrated by bacteriological culturing, the vegetative organisms, on the other hand, could not be revived by this treatment.

The most recent trends in investigations on the mechanism of action of surface-active agents upon bacteria involves studies upon the latter in the presence of human and animal phagocytes. Berry, Starr, and Haller[45] found that certain compounds would at least double the number of bacteria ingested by human neutrophils as compared to control specimens in the absence of the surface-active detergents. The same compounds injected intraperitoneally into mice were also found to cause an elevation of phagocytic activity. Yet these investigators were unable to show any increased protection in *Salmonella typhimurium*-infected mice by the administration of the compounds that were active in the phagocyte-bacteria tests *in vitro*. They explained the latter as being due to the fact that no change in phagocytosis could be observed *in vitro* when the blood cells and bacteria were incubated before they were combined with the detergents.

Nungester and Ames[46] also reported on the influence of surface-active compounds upon phagocytic activity. Their studies reveal that some of the quaternary ammonium salts will materially increase the activity of phagocytes not only *in vitro* but also *in vivo*. Evidence for the latter is found in their data where mice infected with fatal doses of the pneumococcus were protected by treatment with a quaternary ammonium compound. The concentration of the germicide used was not bacteriostatic for the organisms *in vitro.*

There are many references in the literature to the use of "neutralizers," "inactivators," and "antidotal" substances for the evaluation of the antibacterial activity of germicides, disinfectants, "sanitizers," and chemotherapeutic agents. Klarmann[17] has compiled one of the most complete and comprehensive reviews on this general subject, and, for this reason, mention will be made solely of the several substances that have been recommended for the inactivation of the germicidal action of quaternary ammonium compounds.

Domagk[48] was first to call attention to the fact that anionic detergents (soap) will inactivate the surface-active cationic detergents. Soap has been used since then by many investigators to distinguish between the bactericidal and bacteriostatic activity of quaternary ammonium salts (James and Eckfeldt,[49, 50] Pressman and Rhodes,[51] DuBois and Bibblee,[52] *etc.*).

Baker, Harrison, and Miller[53] were able to show that certain synthetic anionic detergents would also inactivate the germicidal properties of cationic detergents. The former compounds have also been used by Rahn,[54] Weber and Black,[55] and others for this purpose. Valko and DuBois[41] reported that bacteria which are apparently "killed" by a quaternary ammonium germicide may be revived by the addition of a high molecular weight anion, provided treatment with the latter occurs within 10 to 30 minutes. Klein and Kardon,[56] on the other hand, were able to demonstrate that "reversal" of quaternary ammonium activity cannot be produced once the organisms have been exposed to germicidal concentrations of the compounds. They found that the addition of an anionic detergent to the treated bacterial suspensions merely interrupted the continued action of the cationic detergents, thereby permitting surviving organisms to grow.

Unlike the ionic reaction between anionic and cationic compounds in the interference of the germicidal action of quaternary ammonium compounds by phospholipids, Baker and her co-workers[53] demonstrated the protective action of lecithin, cephalin, and sphingomyelin on bacteria that were subsequently exposed to lethal concentrations of the cationic germicides. Note was made of the fact that the lipids were ineffective unless they were added to the suspension of organisms just before or at the same time as the quaternary ammonium salts.

Favorable results on the use of lecithin alone or in combination with other substances for the inactivation of cationic germicides have also been reported by Quisno, Gibby, and Foter,[57] Armbruster and Ridenour,[58] and others. The presence of phospholipids or related substances may also account for the inactivating action of the liver broth used by Zeissler and Günther,[59] and the oxgall employed by Klarmann and Wright[60] in their quaternary ammonium investigations.

Brief mention may be made of the studies of Goetchius,[61] in which a sodium salt of a condensed aryl sulfonic acid was used as a cationic inhibitor, and the observations of Lawrence,[62] which indicated that a naphthylene sulfonic acid would neutralize the antibacterial activity of quaternary ammonium compounds.

The most recent trend in the use of "inactivators" for distinguishing bacteriostatic from bactericidal activity of cationic germicides is the addition of the former agents to the sample at the time of collection, plus the incorporation of the same inactivator in the agar in which a determination of the numbers of viable bacteria is to be made (Weber and Black[63]).

Although this concludes this résumé of the literature on the mechanism of action and neutralizing agents for certain surface-active germicides, it is not to be inferred that the review is a complete one. Selection of various publications has been made to indicate the various theories and methods that have been proposed on these subjects. Some of this material will be supplemented and new approaches to methods are envisioned from the papers that follow.

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THE MEANING OF BACTERIOSTASIS, BACTERICIDAL EFFECT, AND RATE OF DISINFECTION

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Traditionally, "bacteriostasis" means prevention of multiplication of bacteria without destroying them, whereas "bactericidal" effect implies forthright killing of the organisms.

A classic example of bacteriostasis is to dip a thread on which test bacteria have been dried into a solution of mercuric chloride. If the thread is cultured immediately in ordinary media, a negative result may be obtained; but if, after exposure to the bichloride, the thread is dipped into a solution of ammonium sulfide, subsequent culture will be positive, showing that in the first test the bacteria were inhibited rather than killed.

When that sort of test is repeated with variations, however, the problem appears less simple. It is found, for example, that prolonged contact with mercuric chloride will eventually kill all of the bacteria. So one must modify his definition to say that, when used briefly, the mercurial is bacteriostatic, but that it is bactericidal when used for a long period of time. Moreover, quantitative tests reveal that, even when the exposure time is short, some of the organisms are killed, while others are merely inhibited. So we must also conclude that our solution of mercuric chloride is at the same time both bacteriostatic and bactericidal.

Bacteriostasis and Bactericidal Effect

By means of some elementary tests, I have tried to reduce this confusing problem of bacteriostasis to simple terms. First, a large number of agar plates, uniformly seeded with approximately equal numbers of colon bacilli, were exposed for various lengths of time to a temperature of 46°C., after which they were incubated at 37°C. The results are shown in TABLE 1. Clearly, the longer the exposure to this relatively slight degree of heat, the fewer the survivors. Although some of the organisms were more resistant to heat than others, eventually all of them died. FIGURE 1 represents the survival time of 3 individual bacterial cells: a sensitive one, an average one, and a tough one. If the test had ended at *a*, all 3 would have survived; if the heat had been continued to *b*, only the tough one would have remained alive; at *c*, all would have been killed.

Actually, in disinfection tests, one deals with large groups or populations of bacteria. If they could be arranged in order of sensitivity and toughness, their survival times might fall into a definite pattern, as in FIGURE 2. That is exactly what the test with heat showed. The values of TABLE 1, plotted against time, produce a regular curve (FIGURE 3). In a test of this sort, therefore, one is faced with several problems. When does the killing begin? When is it complete? What is the pattern between those two points? Or, to put it another way, what is the scatter of sensitivity?

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* Aided by a grant from the Givaudan-Delawanna Company, New York, N. Y.

What is the rate of killing, not for a single cell or a collection of identical cells, but for a large group of cells, the members of which vary tremendously in sensitivity?

The second experiment was like the first, except that cold, 4°C., was used instead of heat. At the end of each test period, the plates were removed from the refrigerator and were placed in the incubator at 37°C. TABLE 2 lists the results. Cold is generally thought to be a bacteriostatic agent *par*

TABLE 1

EFFECTS OF 46°C. ON *E. Coli*

Duration of exposure to heat	*% survivors*
0	100
30 min.	83
2 hrs.	39
10 hrs.	9
17 hrs.	7
1 day	2
2 days	0

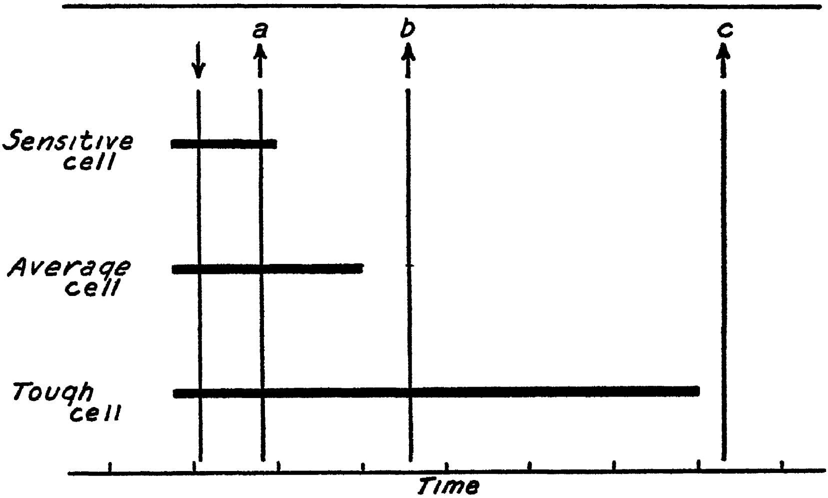

FIGURE 1 Schematic representation of survivial time of three individual bacterial cells exposed to 46°C.

excellence, the one influence above all others capable of producing in bacteria a state of suspended animation; yet this test showed a slowly diminishing number of survivors. Evidently, some of the organisms were sensitive to cold, while others were resistant.

The vital activity of a single bacterium exposed to cold might be represented schematically, as in FIGURE 4. With chilling, the activity is inhibited or depressed. If that inhibition lasts long enough, the cell dies; but if, prior to death, the microorganism is restored to optimum temperature, recovery occurs with resumption of normal activity and function. I say "with resumption of normal activity and function," although in ordinary disinfection tests one actually studies only reproductive activity. One is apt to forget that bacteria have other functions also, such as respiration, metabolism, enzymic activities, hemolysis, toxin production, and infectivity. The effect of inhibition upon these other bacterial functions needs further investigation. There is already some experimental evidence to indicate that they do not necessarily run parallel. In studies of disinfection, we assume too easily that reproductivity measures infectivity.

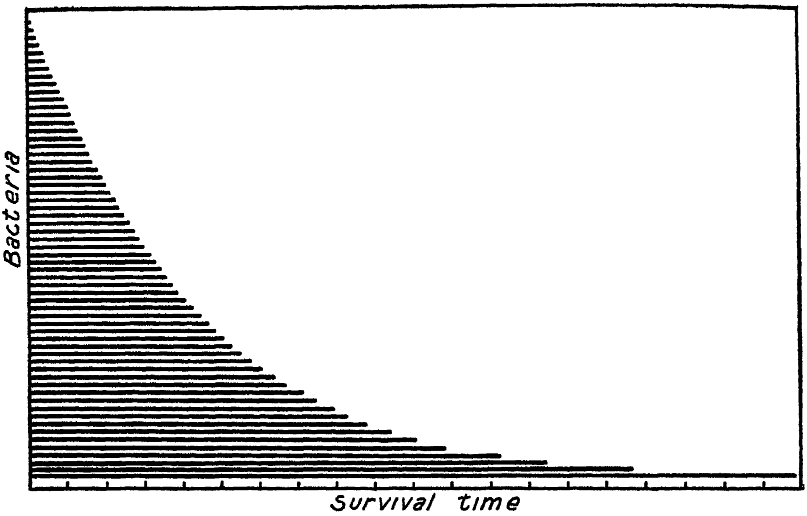

FIGURE 2 Schematic representation of survival time of many bacteria exposed to 46°C.

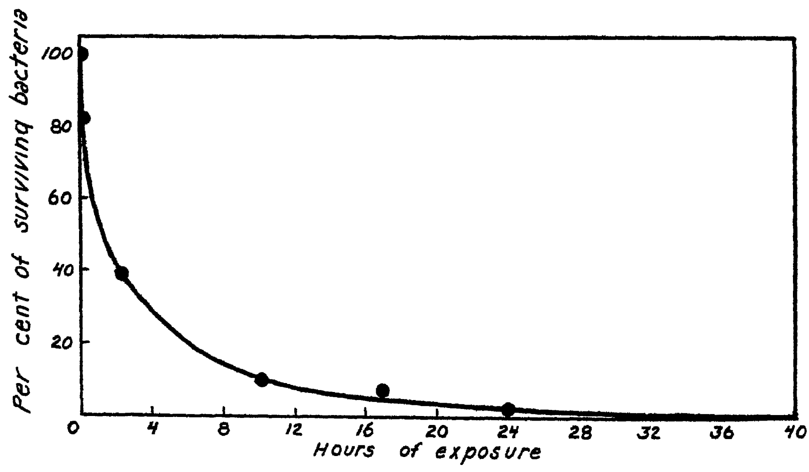

FIGURE 3. Effect of 46°C. on *E coli.*

It is reasonable to suppose that individual bacteria vary in susceptibility to inhibition or depression by cold or any other harmful agent. If the hypothetical test illustrated in FIGURE 5 had ended at the upturned arrow, 1 of the 3 cells would have died, but the other 2 cells would have recovered and would have been able to produce colonies.

In a third experiment, colon bacilli were suspended at room temperature in a weak acid solution—80th normal hydrochloric acid. After varying intervals of time, 1 cc. samples of the suspension were removed, neutralized with appropriate amounts of sodium hydroxide solution, plated, and incubated. The results are shown in TABLE 3. Although lethal action was more rapid, the same general pattern was observed—progressive reduction in the number of living organisms until finally all were killed. In this instance, a chemical neutralizing agent or antidote was used instead of temperature correction (FIGURE 6), but the effect was similar.

TABLE 2
EFFECTS OF 4°C. ON *E. coli*

Duration of exposure to cold	*% survivors*
0	100
2 hrs.	107
10 hrs.	100
1 day	92
5 days	42
10 days	21

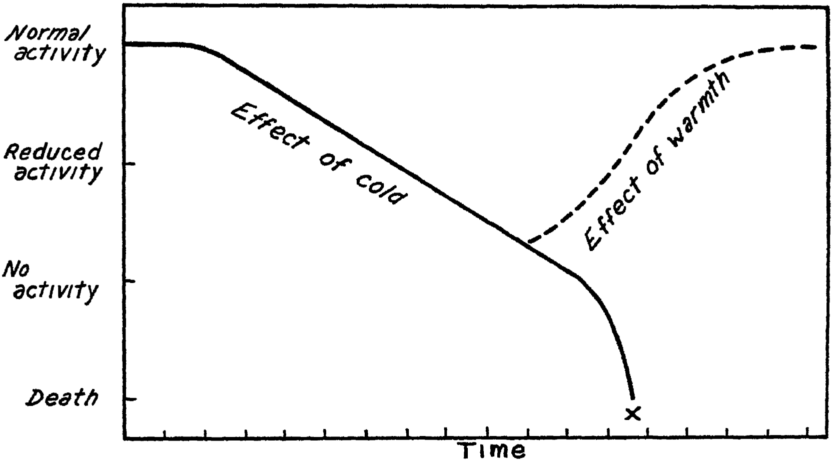

FIGURE 4. Schematic representation of effects of chilling on the vital activities of a bacterial cell

In the next experiment, colon bacilli were suspended in a 1:50,000 aqueous solution of merthiolate at room temperature. Samples of this suspension, taken at appropriate intervals, were neutralized with sodium thiosulfate and cultured in agar plates (TABLE 4). In a parallel study, the antidote was omitted. In this latter set of tests without an antidote, the merthiolate in cultures was diluted to approximately 1 part in 750,000, an amount too small to influence growth in control plates.

It will be seen that 1:50,000 merthiolate, which is supposed to be merely bacteriostatic for colon bacilli, did kill the organisms, reducing their numbers progressively until all were dead, even when an efficient antidote was used prior to plating. When the antidote was not employed, but resort was had to simple dilution, the apparent rate of killing was much more rapid. It was postulated long ago by early workers in this field, perhaps correctly, that the mercurial and the bacterial cell form a union that is not readily dissolved by water. Although merthiolate was not present in these cultures as a whole in sufficient quantities to inhibit growth, it was probably present in or on the implanted test organisms in sufficient concentration to poison them.

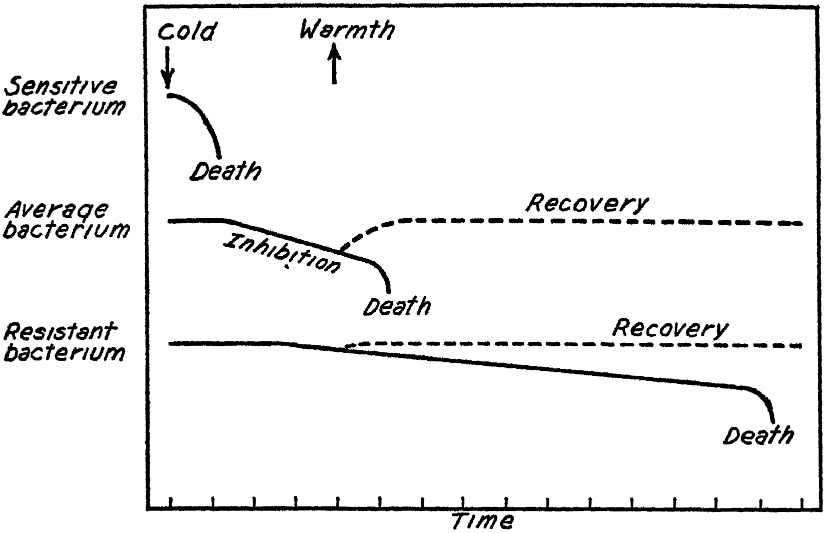

FIGURE 5. Schematic representation of effects of cold on bacterial cells which vary in sensitivity to cold.

TABLE 3
EFFECT OF N/80 HCl ON *E coli*

Duration of exposure to acid	*% survivors*
0	100
1 min.	57
12 min.	23
30 min.	3
2 hrs.	0
9 hrs.	0

These effects may be represented crudely by a diagram (FIGURE 7). If the antidote is applied at the time indicated by the arrow, inactivating the mercury, the bacterium recovers and is capable of producing a colony in an agar plate. Simple dilution will not save the cell, however, although it may prolong its life somewhat. On the other hand, if the antidote is withheld too long, the culture remains sterile, for the cell is already dead.

It should be repeated, however, that one must think not only in terms of a single cell, but also in terms of large groups of bacteria in which there is a gradient of sensitivity to the disinfectant agent—every gradation from extremely sensitive microorganisms to very resistant ones. FIGURE 8 is highly diagrammatic, but it illustrates in the simplest possible way what (in the writer's opinion) happens in the tests just described. The numbers of colonies that grow in plates depend upon the strength of the mercuric solution, the time of contact with the disinfectant, and whether a suitable neutralizing agent is used.

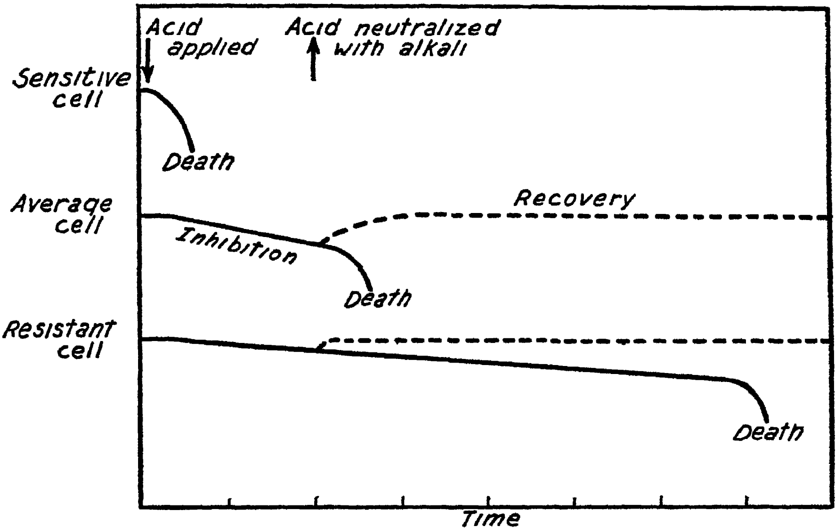

FIGURE 6. Schematic representation of effects of acid on bacterial cells which vary in sensitivity.

TABLE 4
EFFECT OF 1:50,000 MERTHIOLATE SOLUTION ON *E. coli*

Duration of exposure to mercurial	*% survivors*	
	with neutralization	*with dilution*
0	100	100
1 min.	97	4
10 min.	46	1
30 min.	22	0
1 hr.	8	0
2 hrs.	1	0
9 hrs.	0	0

Several other so-called "bacteriostatic" agents have been tested in the same manner, but there is space to mention only one of them. A 1:50,000 aqueous solution of Zephiran,* tested against colon bacilli and using ivory soap as an antidote, gave the results shown in TABLE 5. The same pattern of reduction is observed. This rate of reduction in numbers of survivors is well illustrated if the plate counts are plotted against time on semilogarithmic chart paper (FIGURE 9).

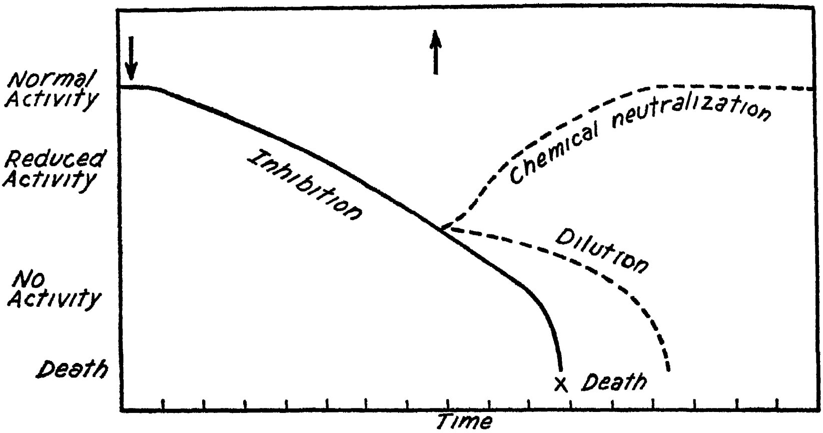

FIGURE 7. Schematic representation of the effect of a mercurial on a bacterium

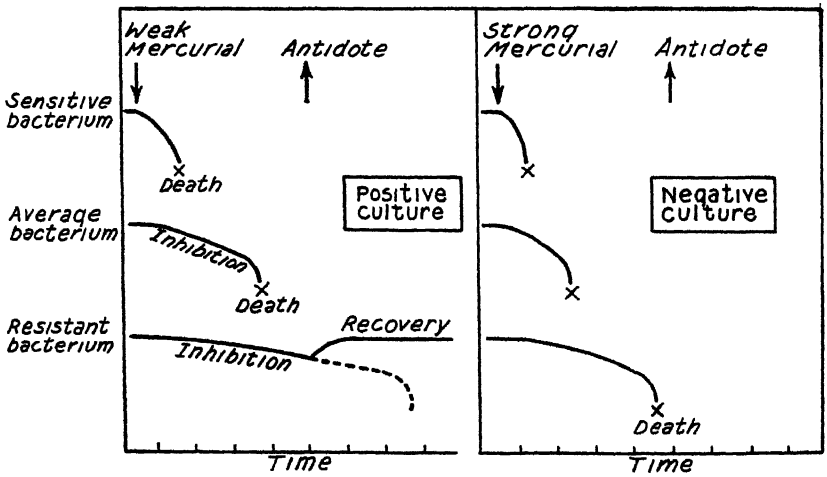

FIGURE 8. Analysis of a test disinfectant action of mercurials

In an experiment like this, the lethal effect, the rate of killing, is the prominent feature. But what about bacteriostasis? What can be said about it? Well, for each bacterium, between the time of first contact with the disinfectant and the time of death, there is a period of inhibition. The cause, nature, and degree of that inhibition are matters which need further elucidation. In some ways, it appears to be similar to shock in animals, in that mild degrees of it may be recovered from spontaneously, but more severe degrees, if not treated properly and in time, lead to a fatal termination. Each bacterial cell of the group seems to have its own degree of susceptibility to both inhibition and death, which we speak of as sensitivity or resistance.

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* Alkyldimethylbenzylammonium chlorides (brand of Winthrop-Stearns, Inc., N. Y.).

TABLE 5
EFFECT OF 1:50.000 ZEPHIRAN SOLUTION ON *E. coli*

Duration of exposure to Zephiran	*% survivors*
0	100
1 min.	92
10 min.	50
30 min.	26
3 hrs.	7
9 hrs.	2
23 hrs.	0

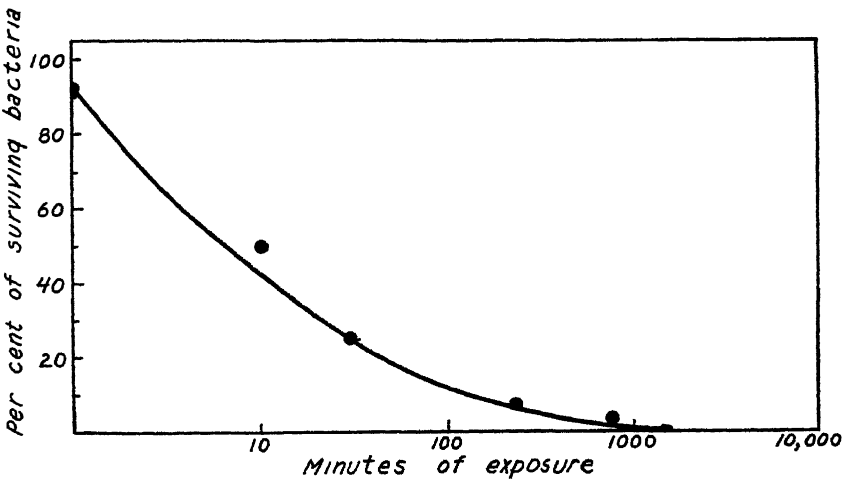

FIGURE 9. Zephiran 1:50,000 aqueous solution effect on *E. coli.*

So far as the writer's experiments have indicated, sensitivity to inhibition and sensitivity to death cannot be separated; they seem to run parallel. Indeed, it may be questioned whether one often sees inhibition free from death, except in so far as one precedes the other. Furthermore, it seems clear that the degree of sensitivity to a given deleterious influence is not an intrinsic quality passed on unaltered to descendants. That is to say, an individual bacterium with a particular degree of sensitivity produces a colony of offspring with various degrees of sensitivity.

So much for inhibition in the individual bacterial cell. It occurs. It can be demonstrated and studied. This may be called bacteriostasis. But when one deals with a large group of test organisms, in which there is a wide range of susceptibility and resistance, the concept of bacteriostasis, as applied to the whole group, becomes less meaningful. All the writer's experimental observations have indicated that, in the group as a whole, inhibition and killing go hand in hand, until sterilization finally occurs. An agent that will inhibit one bacterium will kill an adjacent one, and a third, very resistant neighbor, may remain essentially unharmed. So the question is posed whether it may not be futile and unrealistic to try to differentiate between bacteriostatic and bactericidal agents. It would be somewhat like asking whether the atom bomb at Hiroshima was lethal or simply injurious. It was both. It killed some people quickly; it injured others so severely that they died later; and it injured others less seriously, so that they recovered. Every antibacterial agent is both inhibitory and lethal, often for the individual cell and certainly for the bacterial group as a whole. It is a matter of time. Strong antibacterial agents act more quickly; weak antibacterial agents act more slowly. If the antibacterial action is brought successfully to a stop while there are still some survivors, we get positive cultures; but, if the attempt to terminate the antibacterial activity is delayed until the test organisms are all dead, we get negative cultures.

Evaluation of Disinfectants

How then are we to evaluate disinfectants? What about the tests that have been used all these years? Reference is made again to the schematic representation of FIGURE 2. Somewhere on the chart, the last surviving bacterium is killed, but that is not a sharp end point. On the contrary, as one approaches sterility, one or two colonies per plate keep showing up, and only at long last are all the plates sterile. Nevertheless, that end point is the only part of the test that qualitative liquid-media cultures give reliable information about. The once-popular antiseptic value, the outmoded thread test, the impractical but persistent phenol coefficient test, and efforts to distinguish between bacteriostatic and bactericidal effects are all concerned with that end point, ignoring the very significant effects, inhibitory and lethal, that take place in the test before that time.

If antibacterial agents are to be evaluated at all on the basis of *in vitro* tests, it would seem better to employ some carefully controlled and standardized quantitative test. The writer has experimented with a test of that sort. TABLE 6 indicates the results obtained when different concentrations of ethyl alcohol are tested for various lengths of time against suspensions of *Staphylococcus albus*. But the only information that any *in vitro* method can give is the effect of a particular disinfectant solution on the specific test organism under the conditions of the test. It is not justifiable, and it may be quite misleading, to assume that, because a disinfectant is effective *in vitro* under experimental conditions, it is equally effective under conditions of clinical use.

Many investigators, who have focused attention exclusively on the end point of sterilization in tests, have underestimated the inhibitory and slowly destructive action of minute quantities of antibacterial agents, especially in those tests where dilution rather than chemical neutralization are relied upon to terminate the effect of the disinfectant. I have found, for example, in working with *S. albus* and G-11,* that 1 part of G-11 in 5 million in agar will result in negative cultures, and that amounts greater than 1 part in 20 million will reduce the number of colonies. Failure to recognize that amazing effect and the lack of an adequate antidote for the compound, plus its characteristic insolubility in water, have led many workers, because of negative cultures, to conclude that G-11 acts more quickly and strongly than it actually does.

Fortunately, during recent years, the trend has been toward developing methods which test disinfectants under conditions of actual use. The so-called "infection-prevention" tests may be open to justifiable criticisms, but they also have real value. A closer approach to true evaluation of disinfectants under conditions of actual use lies in the serial basin hand-washing method. Its technic is tedious, but properly performed it avoids most of the pitfalls of other tests and gives valuable and relatively trustworthy results. FIGURES 10 to 14 show graphically the results obtained by means of this test when some of the more commonly used skin disinfectants are employed. It will be observed that their effects are expressed in terms of the rate of reduction of the cutaneous flora.

TABLE 6

GERMICIDAL EFFECT OF ETHYL ALCOHOL ON *S. albus**

Duration of contact between germicide and bacteria	*Concentration of alcohol—per cent by weight*									
	10%	*20%*	*30%*	*40%*	*50%*	*60%*	*70%*	*80%*	*90%*	*99%*
0 (control)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1 sec.	108.3	104.5	80.2	71.4	68.8	59.5	27.1	29.9	68.8	61.9
10 sec.	86.7	94.6	65.8	77.6	41.2	34.9	26.6	26.6	37.3	60.7
30 sec.	106.5	92.7	50.2	47.2	35.7	18.4	21.2	34.1	48.2	48.7
1 min.	127.5	91.1	49.8	35.5	22.7	9.3	18.6	14.9	54.4	46.8
5 min.	108.4	89.9	12.2	14.1	15.5	12.2	21.7	24.4	41.9	26.2
10 min.	90.4	73.6	24.6	14.7	9.9	13.5	23.4	20.1	46.0	41.8

* The numbers in the table indicate the per cent of test organisms surviving after contact with alcohol.

When 2 per cent G-11 soap is used for 5 or 10 minutes in a single scrub, the bacterial flora of the skin is not reduced any more rapidly than when scrubbing is carried out with ordinary soap. But if G-11 soap is used several times a day, every time the hands are washed, for 4 or more days, the resident bacterial flora is reduced to approximately 5 per cent of its ordinary size (FIGURE 10). That low flora appears to be maintained as long as the G-11 soap is used faithfully, but, as soon as its use is discontinued, the ordinary flora begins to be re-established.

Aqueous Zephiran solution has relatively little power to reduce the bacterial flora. Tincture of Zephiran is more effective; but, as FIGURE 11 suggests, the increase in disinfectant action is due mainly to the alcohol acetone solvent.

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* 2,2′-dihydroxy-3,5,6,3′,5′,6′-hexachlorodiphenylmethane (Givaudan-Delawanna Company, N Y.).

FIGURE 12 shows the results obtained with some of the commonly used mercurials. All of the solutions were applied with light gauze friction, much as one paints a patient's skin in preparation for operation. It can be seen that the best of these solutions has reduced the flora by less than half in 3 minutes. The effect of washing in a similar manner with soap and water is included for comparison. It is often stated that soap and water are as effective disinfecting agents for the skin as anything else. Obviously that is not correct. Soap removes grease, dirt, and loosely attached contaminating bacteria, and for that reason its use is important; but it does not reduce the resident bacterial flora efficiently.

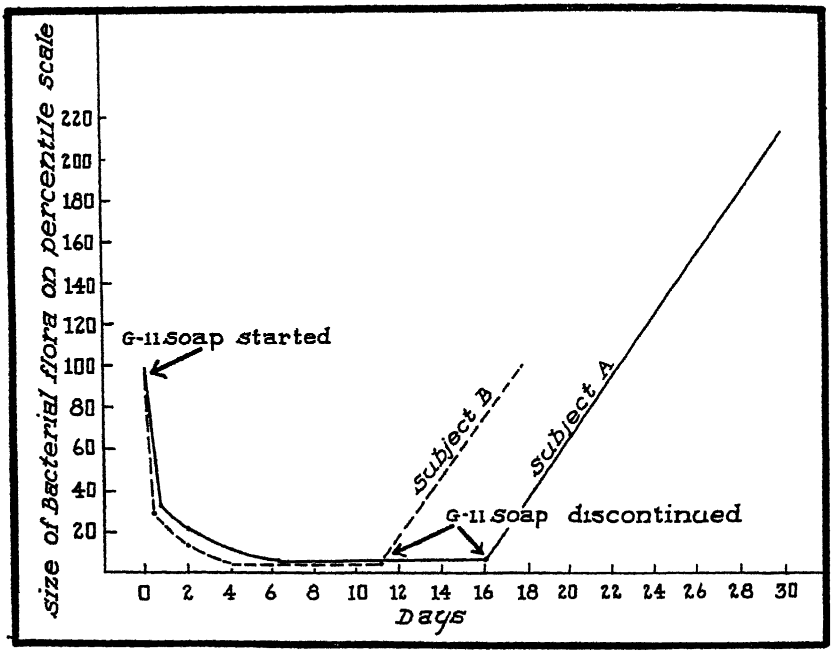

FIGURE 10. Effects of frequent daily use of 2 per cent G-11 soap upon the flora of the hands and arms.

Alcoholic solutions of these same mercurials are more effective than the corresponding aqueous solutions (FIGURE 13). The difference is due mainly, however, to the germicidal action of the solvent. Indeed, the disinfectant action would be greater if the solvent was used without the mercurials.

The best results were obtained with alcohol and iodine (FIGURE 14). Seventy per cent ethyl alcohol by weight is an excellent skin disinfectant. Addition of one or two per cent iodine to it increases its action slightly. As a rule, these iodine solutions do not irritate the skin. They spread evenly, dry slowly, and in 3 minutes reduce the resident flora to less than 10 per cent of its original size. Lugol's solution and 7 per cent tincture of iodine

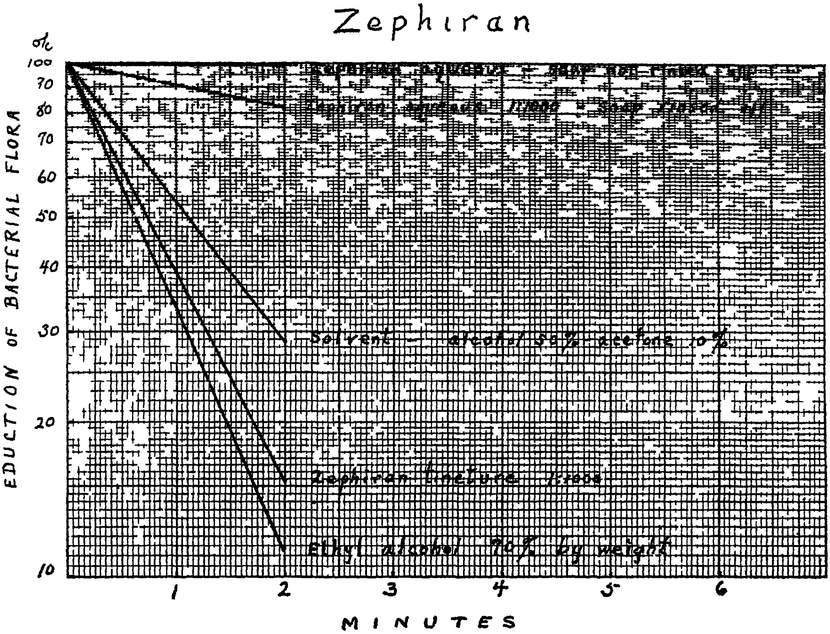

FIGURE 11 Effects of Zephiran and of alcohol upon the bacterial flora of the hands and arms Each solution was washed in without friction for exactly 2 minutes

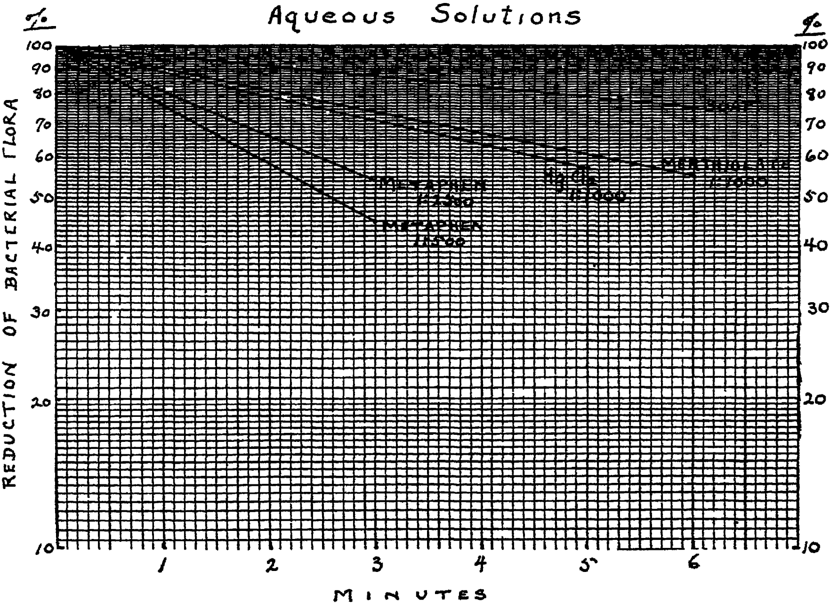

FIGURE 12 Effects of aqueous solutions of mercurials upon the bacterial flora of the hands and arms The various preparations, washed in for the lengths of time indicated, at 25°C, were followed immediately at the end of the test period by an antidote

(U.S.P.) are even more powerful, but they are too irritating and even harmful to the skin for routine use.

Comment

The final answer to the problem of evaluation of disinfectants is an elusive one, but progress is being made in that direction. In conclusion, some general principles should be emphasized which can serve as a guide in further investigations and conclusions.

(1) Terms such as *germicide*, *antiseptic*, *disinfectant*, and *bacteriostasis* need to be more accurately defined and used.

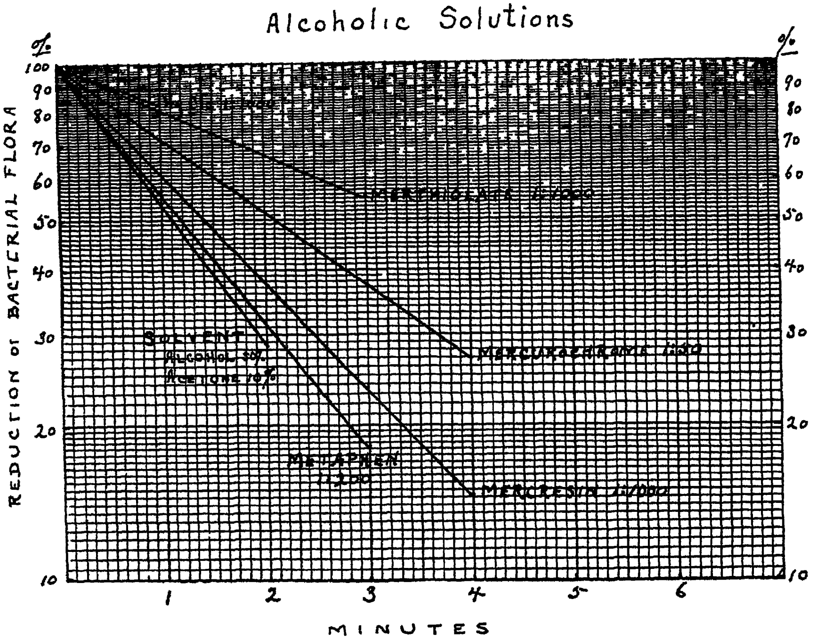

FIGURE 13 Effects of various mercurials in alcoholic solution upon the bacterial flora of the hands and arms All tests were made at 25°C and were followed by suitable antidotes

(2) Experimental results should be considered valid only for the conditions of the experiment. Uncritical transfer of results from purely laboratory tests to clinical use may be dangerously misleading.

(3) There is need for greater standardization of all tests that are adopted, with rigid control of variables.

(4) In general, quantitative tests are to be preferred to purely qualitative ones.

(5) In so far as possible, methods should be employed which test disinfectants under conditions of actual use. Disinfectants should be tested specifically; for example, for their ability to disinfect healthy skin, contaminated skin, instruments, excreta, secretions, wounds, *etc.*

(6) Effective chemical antidotes should be employed in all tests except in the case of those disinfectants, such as alcohol, in which dilution can be shown to cause immediate and complete cessation of the antibacterial activity. At the same time, it must be kept in mind that in practice antidotes are not deliberately used. A mercurial on the skin, for instance, is admittedly more effective as a surface disinfectant if it is not followed by an antidote. Since, in preparation for surgery, mercurials are used without antidotes, why employ antidotes in our tests? The answer is that body tissues contain sulfur compounds and other reducing substances which serve more or less effectively as antidotes. That is another aspect of the problem that needs further investigation. In those instances where chemical antidotes are unknown or are imperfect, and where simple dilution alone is not fully effective, due caution must be exercised in passing judgment on the true value of the disinfectants under investigation.

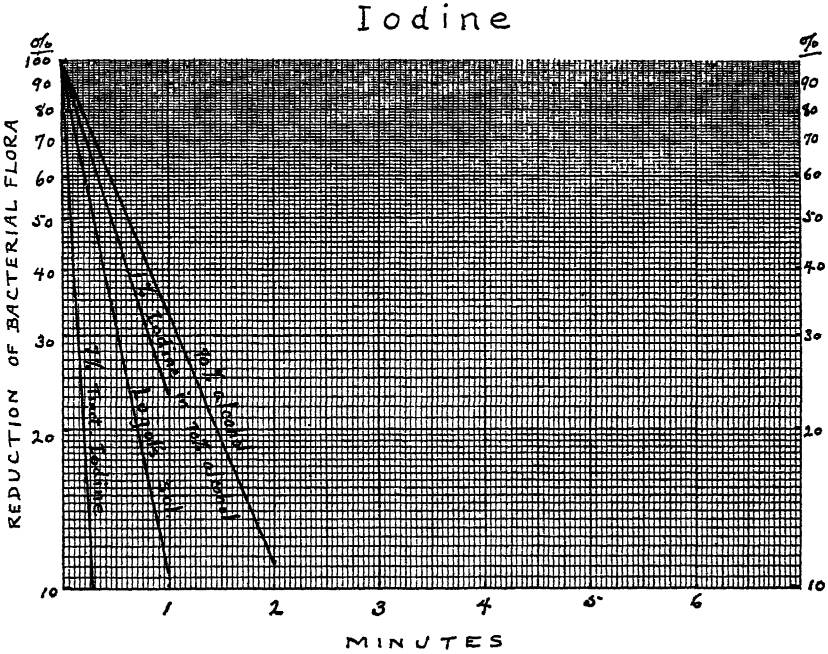

FIGURE 14. Effects of 70 per cent ethyl alcohol and various solutions of iodine upon the bacterial flora of the hands and arms. All tests were made at 25°C. In the case of iodine, appropriate antidotes were employed.

Summary

Studies of bacteriostasis, bactericidal effect, and rate of killing, using quantitative bacteriological methods, strongly suggest that the time-honored concepts of these phenomena are inadequate. Disinfection appears to be a more complex matter than has been generally thought.

An attempt is made to analyze and elucidate "bacteriostasis." A hypothesis is advanced which appears to explain the phenomenon more satisfactorily than the conventional view of temporary-inhibition-without-killing. Experiments are cited which lend support to the hypothesis.

Various tests which in the past have been used to evalute disinfectants are reviewed critically in the light of this new concept. Consideration is given also to important guiding principles to be used in establishing more satisfactory standard methods of measuring antibacterial activity.

THE INTERACTION OF SURFACE ACTIVE AGENTS AND PROTEINS

By Harold N. Glassman*

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Upon interaction with systems of biological interest, surface active compounds have exhibited marked effectiveness in low concentrations with phenomena such as precipitation, complex formation and denaturation of proteins, cytolysis of cells, destruction of microorganisms, and inactivation of viruses as examples.[1, 2] There exists a relatively meager body of fundamental knowledge to explain these phenomena. This is due partly to the emphasis on the applied phases of surface active agent research, stemming, in the biological field, from the demonstration by Domagk[3] of the high bactericidal efficiency of these compounds. Partly responsible, also, is the lack of readily available, purified surface active compounds for research. This, again, is a reflection of the emphasis on application of surface active agents, which is as true in general industrial fields as it is in the more restricted biological field.

Our interest lies in the interaction of surface active agents and proteins, especially those possessing a highly characteristic biological activity, as a basis for understanding the effects of surface active agents on more complicated biological systems. In this paper, investigation of the interaction of surface active agents with crystalline bovine plasma albumin and highly purified preparations of both types A and B botulinum toxins will be reported. Precipitation and redispersion phenomena have been studied in the case of all these proteins and, in addition, studies of the alteration of biological activity have been made in the case of the toxins. Similar work with other biologically important systems (crystalline egg albumin, viruses, and enzymes), referred to briefly here, will be presented in greater detail elsewhere.

Previous studies in the literature have indicated that interaction of surface active agents and proteins may result in precipitation, redispersion, complex formation, and denaturation.[2] Such factors as the ionic nature of the surface active agent, the length of its paraffinic carbon chain, the pH, and the mass ratio of the surface active agent to protein have been found to be governing variables. While many of these investigations have been qualitative in nature, some of the more recent studies, such as those of Putnam and Neurath,[4] on the horse serum albumin-dodecyl sulfate system, and Lundgren, *et al.*,[5, 6] on the egg albumin-alkyl benzene sulfonate system, have been quantitative in approach. Recent thermodynamic studies[7, 8] of the plasma albumin-dodecyl sulfate system have attempted to evaluate the number of groups available on this protein for binding dodecyl sulfate and the intensity with which this binding takes place. These studies are valid only under conditions where neither micelle formation nor denaturation occurs and, therefore, have been carried out at concentrations below those ordinarily of interest in the biological applications of surface active agents. These studies have all been confined to an anionic surface active agent. The cationic compounds, however, are often of much greater interest biologically. In the work presently being reported, anionic, cationic, and nonionic surface active agents have been utilized.

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* The review of F. W. Putnam[2] materially influenced the organization of these studies. It is a pleasure to record the generosity of Dr. Putnam in making available the manuscript of his review some twenty months prior to publication.

Methods and Materials

The crystalline bovine plasma albumin was prepared by the alcohol fractionation method of Cohn[9] and obtained from a commercial source. The types A and B botulinum toxins were highly purified preparations[10, 11] assaying 9.5×10^{-9} mg. N per mouse LD_{50} for the type A and 12.6×10^{-9} mg. N per mouse LD_{50} for the type B.* A single purified preparation of each toxin, kindly donated by Dr. Carl Lamanna, was used throughout this investigation. Numerous preliminary experiments were conducted with toxin solutions of a lesser degree of purity. The results of these experiments are in complete agreement with those about to be reported but will not be presented in detail. We should like to point out that, while these two toxins are similar toxicologically, they differ in many of their chemical, physical, and immunological properties and represent two different proteins.[10–12]

Toxicity assays of these toxins were made by the intraperitoneal injection into 15 to 20 gram mice of 0.5 ml. quantities of appropriately diluted solutions, with an observation period of 4 days. Dilutions were made with a solution consisting of 0.2 per cent gelatin buffered at pH 6.8 by 1 per cent sodium phosphate. Nitrogen analyses were performed by the micro-Kjeldahl method. Conversion of nitrogen content to protein mass was made, using values of 16 per cent for the nitrogen analysis of the crystalline bovine plasma albumin,[9] 16.29 per cent for the type A,[26] and 15.5 per cent for the type B[11] botulinum toxins.

The bulk of the experimental work was performed with a single representative each of an anionic, cationic, and nonionic surface active agent. Lorol, the anionic surface active agent, is a straight chain alcohol sulfate, with the C_{12} and C_{14} homologs present in the ratio of 80 to 20 on the basis of the alcohols used for sulfation.[14] Less than 0.2 per cent unreacted alcohol or extraneous salts were present. This material was made available through the courtesy of the du Pont Company. Cetyl dimethyl benzyl ammonium chloride, which will be referred to hereafter as CDBAC, was used as the cationic surface active agent. It is more than 98 per cent pure, with the C_{18} homolog as the chief impurity.[15] Triton A-20, a polymeric polyether alcohol[16] made available by the Rohm and Haas Company, was used as the nonionic surface active agent. We had a special interest in this compound, since we have shown it to be quite free from deleterious biological effects, as exemplified by low toxicities and meager hemolytic activity.[17]

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* The term LD_{50} indicates the dose lethal for 50 per cent of the mice injected and was calculated by the method of Reed and Muench.[13] MLD is used to indicate the minimal dose of toxin lethal to all the mice injected.

Certain confirmatory experiments were performed with other surface active agents, which will be mentioned as this work appears in the text.

Results

Precipitation of Protein as a Function of pH. A rapid survey of the effect of alteration of pH on the precipitation of these proteins by surface active agents was made by titrating solutions containing approximately 0.2 mg. protein per ml. with 0.05 per cent solutions of the surface active agents. Prior to the titration and after each addition of the given surface active agent, the transmittance of these solutions was measured in a Klett-Summerson photoelectric colorimeter (#42 filter). The course of a typical precipitation may be described by an optical density gradually increasing to a maximum value. As the surface active agent is added beyond this point, redispersion of the precipitate occurs, with consequent decrease in optical density. Inclusion of all these points at each pH would make too complex a figure, so we have resorted to portraying only the maximal change in optical density.

FIGURE 1 illustrates the interaction of crystalline bovine plasma albumin and surface active agents over the pH range 3 to 10. The isoelectric point of this protein at 0.1 ionic strength is pH 4.71.[18] It will be seen that on the acid side of the isoelectric point only the anionic compound caused precipitation. In the pH range of 5 to 8, neither of the ionic surface active agents caused the plasma albumin to precipitate. At pH values more alkaline than 8, only the cationic surface active agent was effective in causing precipitation.

In FIGURE 2, we have the results of a similar experiment utilizing the type B botulinum toxin. It should be noted that the pH range used in this experiment is much narrower than that studied with crystalline bovine plasma albumin (FIGURE 1). As yet, no accurate determination of the isoelectric point of this protein is available. However, minimal solubility seems to be exhibited at pH values about 5.5. It will be seen that, at pH levels on the acid side of the isoelectric region, precipitation is attained with the anionic compound and there is an enhancement of dispersion by the cationic compound. In the isoelectric region, both ionic compounds yield a precipitate. On the alkaline side of the isoelectric range, the cationic surface active agent causes the toxin to precipitate, while, if anything, there is a slight enhancement of dispersion upon addition of the anionic compound.

Data similar in character have been obtained with the type A botulinum toxin, crystalline egg albumin, and several purified enzymes.[17] In the case of all these proteins, titration with the nonionic surface active agent did not produce precipitation at any pH or at any concentration used. We have, therefore, omitted the nonionic compounds from representation in FIGURES 1 and 2.

Quantitative Protein Precipitation. Quantitative studies of the precipitation of these proteins by ionic surface active agents were carried out on the acid and alkaline sides of their isoelectric points. Tenth molar acetate buffer or phosphate plus NaOH were used as solvents. In these experiments, a given amount of protein was interacted with a graded series of concentrations of surface active agent and the interaction mixture allowed to stand 1 to 3 hours. It was then centrifuged and the supernatant analyzed for nitrogen content.

The quantitative study of the interaction of crystalline bovine plasma albumin on the acid side of the isoelectric point and Lorol is represented in FIGURE 3. When the per cent protein precipitated is plotted as a function of the mass ratio of surface active agent to protein, three distinct regions may be observed. The first, including all mass ratios of surface active agent to plasma albumin up to 0.22, may be called the region of protein excess with incomplete precipitation of the plasma albumin. From a mass ratio of 0.22 to 0.41, the equivalence zone, complete precipitation of the plasma albumin by the Lorol is obtained. At mass ratios of the reactants above 0.41, the surface active agent is in excess, and partial or complete dispersion of any precipitate occurs. It will be noted that three different concentration levels of protein were used with identical results, indicating the stoichiometric character of the interaction. For comparison of these results using crystalline plasma albumin of *bovine* origin with similar experiments carried out on albumin of *equine* origin, we have incorporated into this figure by dashes the data of Putnam and Neurath[2, 4] covering a similar concentration range,

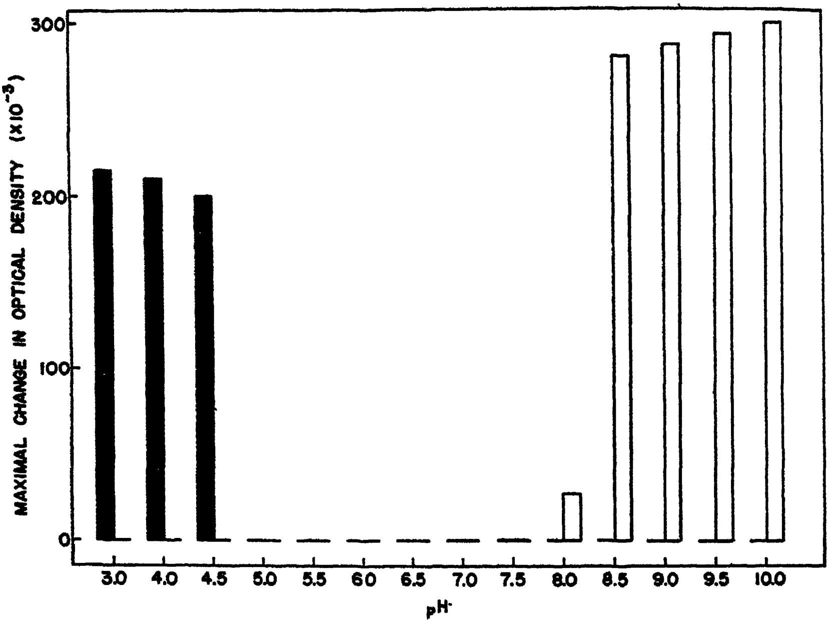

FIGURE 1. The interaction of crystalline bovine plasma albumin and surface active agents as a function of pH. The solid bars represent interaction with the anionic compound, Lorol, and the open bars represent interaction with the cationic compound, cetyl dimethyl benzyl ammonium chloride (CDBAC).

Good agreement between the results of these two sets of experiments is evident.

Similar curves are available for crystalline egg albumin and several crystalline enzymes.[17] Quantitative precipitation experiments with the botulinum toxins and Lorol showed entirely analogous results, with the exception that the maximal precipitation attained varied from 70 to 80 per cent, as contrasted to the 100 per cent just described. The mass ratios for this optimal precipitation were 0.12 to 0.36 for the type A toxin and 0.27 to 0.55 for the type B toxin.

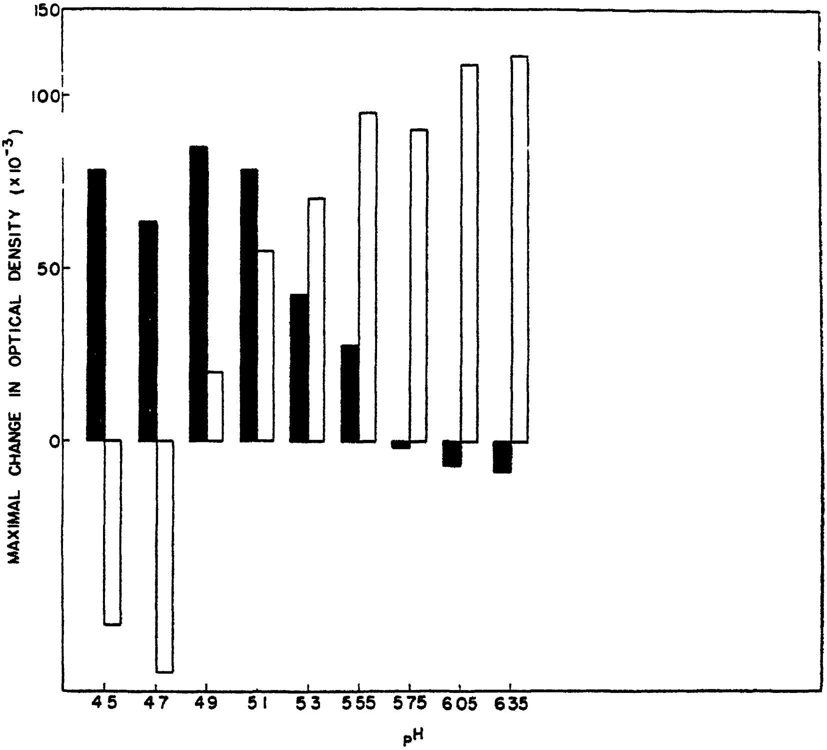

FIGURE 2. The interaction of type B botulinum toxin and surface active agents as a function of pH. The solid bars represent interaction with the anionic compound, Lorol, and the open bars represent interaction with the cationic compound, cetyl dimethyl benzyl ammonium chloride (CDBAC).

The quantitative precipitation experiments that were carried out on the alkaline side of the protein isoelectric point utilized the catonic surface active agent, CDBAC. There is a small error introduced into these estimations, due to the fact that the CDBAC contains 3.5 per cent nitrogen. It will be seen (FIGURE 4), however, that the results obtained are entirely similar to those obtained on the acid side of the protein isoelectric point, with incomplete precipitation in the region of protein excess, with an optimal precipitation in the equivalence zone, and with partial or complete redispersion of

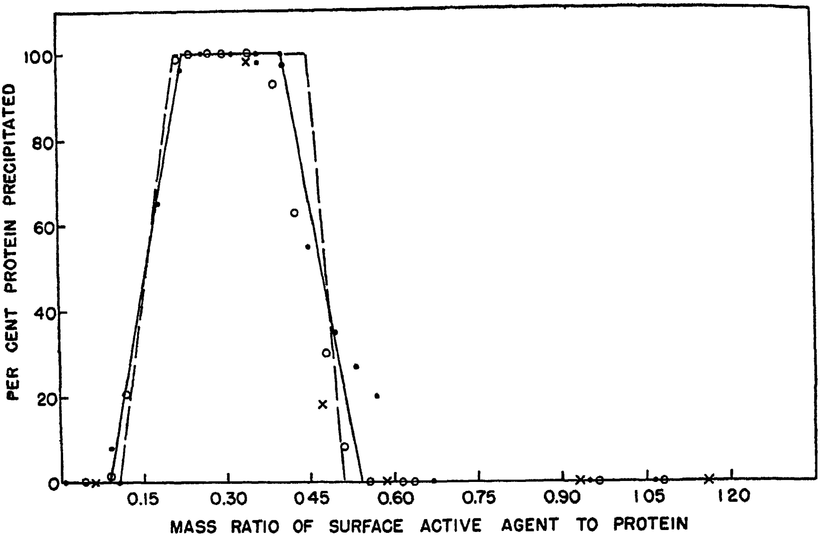

FIGURE 3. The interaction of crystalline bovine plasma albumin on the acid side (pH 4.4) of the isoelectric point and the anionic compound, Lorol. Albumin concentration levels in mg. per ml are: ● = 4.2512, ○ = 8.4612, and × = 17.1890. The dashes represent data on the interaction of crystalline *horse* serum albumin and sodium dodecyl sulfate.[2]

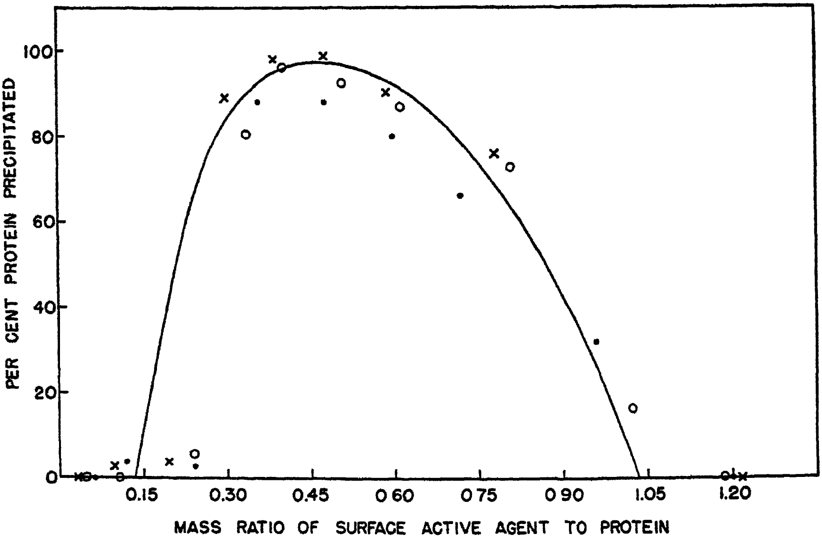

FIGURE 4. The interaction of crystalline bovine plasma albumin on the alkaline side (pH 9) of the isoelectric point and the cationic compound, cetyl dimethyl benzyl ammonium chloride (CDBAC). Albumin concentration levels in mg. per ml. are: ● = 4.3542, ○ = 8.8876, and × = 18.3350.

the precipitate in the region of surface active agent excess. Again, use of three different concentration levels of protein yielded identical results.

Influence of Surface Active Agents on Toxin Activity. While precipitation as visible evidence of complex formation is restricted to conditions where the reactants bear an opposite net electrostatic charge, it should not be inferred that there is no interaction when the reactants are similarly charged (*e.g.*, a protein on the alkaline side of its isoelectric point in interaction with an anionic surface active agent). Electrophoretic,[5, 19] diffusion,[20, 21] viscosity,[21] and dialysis equilibrium[7, 8] studies have indicated that definite complexes may be formed when the protein and surface active agent both bear the same net electrostatic charge. Indeed, it has been demonstrated that the mass ratios of reactants which are required for optimal precipitation of crystalline horse serum albumin on the acid side of its isoelectric point by dodecyl sulfate are identical with the mass ratios of the same reactants required for electrophoretic demonstration of complex formation when the protein is on the alkaline side of its isoelectric point.[4, 19] In view of these facts, our studies of the influence of interaction with surface active agents on the biological activity of botulinum toxin were carried out at pH values on both sides of the toxin isoelectric point.

TABLE 1
INTERACTION OF TYPE B BOTULINUM TOXIN ON THE ACID SIDE (pH 3) OF THE ISOELECTRIC POINT AND LOROL

Mass ratio SAA/toxin*	*% Toxin precipitated*	*Toxicity (MLD × 10^8) per ml. of reconstituted precipitate*	*Toxicity (MLD × 10^8) per ml. of the supernatant fluid*
Control (no SAA)	—	—	1,000
0.07	6	1	10
0.14	33	<1	<1
0.21	70	<1	<1
0.35	73	<1	<1
0.49	77	<1	<1
0.70	12	<1	<1
0.84	9	<1	<1
3.50	0	—	<1

* SAA = surface active agent.

Interactions between toxin and surface active agents were carried out in 0.1 M HCl (pH 1), 0.1 M acetic acid (pH 3), or 0.1 M phosphate buffer* (pH 7.0 or pH 7.2). Interaction was allowed to proceed 1 to 3 hours at room temperature. In situations where no precipitate resulted, this interaction mixture was assayed directly for toxicity. Where precipitates did occur, they were separated by centrifugation and the supernatant removed by filtration through washed glass wool. These precipitates were reconstituted to the original volume of the toxin solutions with 0.1 M phosphate buffer at pH 7. Both the reconstituted precipitates and the supernatants were assayed for toxicity. The term "complete inactivation" is used to indicate 99+ per cent loss in toxicity.

When type B botulinum toxin on the acid side (pH 3) of the isoelectric point is allowed to interact with Lorol, precipitation is observed (TABLE 1). It will be seen that complete inactivation of the toxin is evident at a mass ratio of surface active agent to toxin of 0.14. At this mass ratio, only 33 per cent of the toxin has been precipitated. It will be noted that at high mass ratios (*e.g.*, 3.50) there is no precipitation, but inactivation is still complete.

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* Only sodium salts were used, due to the relative insolubility of the potassium salts of certain alkyl sulfates.[4]

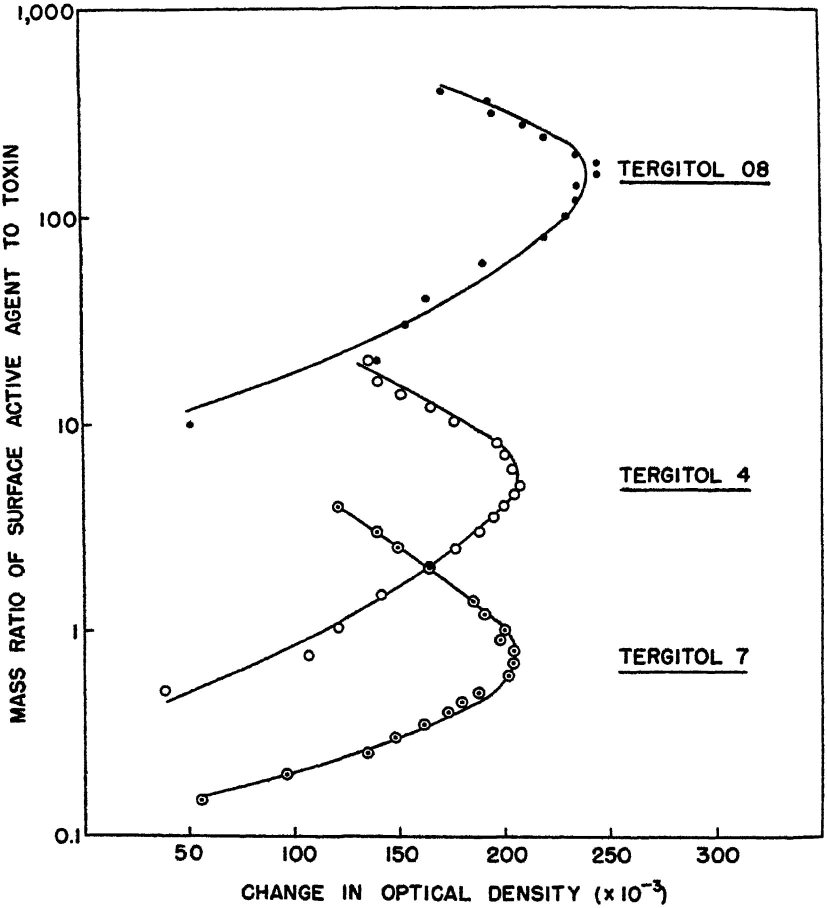

FIGURE 5. Titration of type B botulinum toxin on the acid side (pH 3) of the isoelectric point with three anionic surface active agents of graded paraffinic chain length.

Study of the type A toxin on the acid side (pH 1) of the isoelectric point, in interaction with the same anionic surface active agent, yielded a similar picture, with complete inactivation at a mass ratio of Lorol to toxin of 0.16.

An observation which seems to be universally made in any homologous series of surface active agents is a gradation of properties with increasing carbon chain length. We have found this to be true for these precipitation and inactivation studies with the botulinum toxins. When either decyl or octyl sulfate is substituted for Lorol, precipitation, redispersion, and inactivation are again obtained in an analogous manner, except that increasing amounts of the surface active agent are required as its chain length decreases.

A graphic demonstration of the gradation in efficiency obtained with a changing paraffinic chain length may be observed (FIGURE 5) in precipitation studies with three different Tergitols.* Each of these is a similar branched-chain anionic surface active agent, with the 08 compound having the shortest chain length and the 7 compound having the longest. This graph represents the plot of the changes in optical density as a given amount of type B botulinum toxin was titrated with these compounds. It will be noted that all three curves are similar in demonstrating an increase in optical density to a maximal value, with redispersion evident as additional Tergitol is added beyond this maximal value. The mass ratio of Tergitol to toxin for optimal aggregation decreases, however, as the chain length of the Tergitol grows longer, so that the maximal change in optical density is obtained at a mass ratio of approximately 180 for Tergitol 08 (which has 8 carbons), 5 for Tergitol 4 (which has 14 carbons), and 0.8 for Tergitol 7 (which has 17 carbons).

TABLE 2

INTERACTION OF TYPE B BOTULINUM TOXIN ON THE ACID SIDE (pH 3) OF THE ISOELECTRIC POINT AND SANTOMERSE D

Mass ratio SAA/toxin*	*% Toxin precipitated*	*Toxicity (MLD × 10^3) per ml. of reconstituted precipitate*	*Toxicity (MLD × 10^3) per ml. of the supernatant fluid*
Control (no SAA)	0	—	3,160
0.09	0	—	1,000
0.29	19	<10	<10
0.97	83	<10	<10
2.91	6	<10	<10
9.70	0	<10	<10

* SAA = surface active agent.

These results, obtained using the surface active agents which we have chosen as representative, might be considered to have some specific relationship to the individual agents used. We have, therefore, at various stages in our work, carried out experiments with surface active agents of different structures. For example, we have carried out precipitation and inactivation studies with a decyl benzene sulfonate (Santomerse D*).

It will be seen (TABLE 2) that in all respects (precipitation, redispersion, and inactivation) there is a complete analogy between these Santomerse experiments and the Lorol experiments previously discussed.

When the interaction of toxin and Lorol is now studied on the alkaline side of the toxin isoelectric point (where, of course, we do not get any precipitation), we observe (TABLE 3) that, although complete inactivation is obtained, it requires a much higher concentration of Lorol than was required at pH levels where the Lorol precipitated the toxin. Thus, at pH 7, a mass ratio of Lorol to toxin of 2.64 was necessary for inactivation, as compared with a mass ratio of 0.14 at pH 3. A study of the inactivation of the type A toxin on the alkaline side of its isoelectric point yields entirely similar results

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* Product of Carbide and Carbon Chemicals Corporation.

* Product of Monsanto Chemical Company.

TABLE 3
INTERACTION OF TYPE B BOTULINUM TOXIN ON THE ALKALINE SIDE (pH 7) OF THE ISOELECTRIC POINT AND LOROL

Mass ratio SAA/toxin*	*Toxicity (MLD × 10^3) per ml. of the mixture*
Control (no SAA)	1,000
0.09	1,000
0.18	1,000
0.35	1,000
0.53	1,000
0.88	316
1.32	10
1.76	10
2.64	<10
3.52	<10

* SAA = surface active agent

TABLE 4
INTERACTION OF TYPE B BOTULINUM TOXIN ON THE ALKALINE SIDE (pH 7) OF THE ISOELECTRIC POINT AND CDBAC

Mass ratio SAA/toxin*	*Toxicity (MLD × 10^3) per ml. of reconstituted precipitate*	*Toxicity (MLD × 10^3) per ml. of the supernatant fluid*
Control (no SAA)	—	1,000
0.03	<10	1,000
0.06	<10	10
0.12	<10	<10
0.18	<10	<10
0.30	<10	<10
0.60	<10	<10
1.20	<10	<10
3.00	<10	<10

* SAA = surface active agent

This decreased efficiency in inactivation of the toxin at pH 7 as compared with pH 3 is shared also by the decyl benzene sulfonate.

Thus, it is evident that the anionic surface active agent is most efficient in inactivating botulinum toxin at a pH level on the acid side of the toxin isoelectric point. Under these conditions, the two reactants bear opposite net charges and precipitation may be observed at certain mass ratios.

The initial experiments to be described utilizing the cationic surface active agents will be those on the alkaline side of the toxin isoelectric point. Under these circumstances, the net charge on the reactants is of unlike sign and precipitation is observed at appropriate mass ratios. When the type B toxin on the alkaline side (pH 7) of the isoelectric point is allowed to interact with CDBAC, complete inactivation of the toxin is evident at a mass ratio of CDBAC to toxin of 0.12 (TABLE 4). Although the precipitate is largely redispersed at mass ratios of CDBAC to toxin above 1, the toxin remains inactivated. The corresponding experiment, utilizing the type A toxin on the alkaline side (pH 7.2) of its isoelectric point, shows complete inactivation of the toxin at mass ratios of CDBAC to toxin of 0.51 or greater. Use of cetyl pyridinium chloride as an alternate surface active agent structure yields a similar picture of precipitation and inactivation of the botulinum toxins on the alkaline side of their isoelectric points.

A far different picture is evident when the interaction of CDBAC and toxin is studied on the acid side of the toxin isoelectric point. No precipitation is observed under these conditions. When CDBAC and the type B toxin are interacted at pH 3, only partial inactivation of the toxin is evident even at mass ratios of CDBAC to toxin as high as 50 (TABLE 5). This should be contrasted with the complete inactivation obtained at pH 7 using the same surface active agent at a mass ratio of surface active agent to toxin of 0.12 (TABLE 4). This relative inefficiency in inactivating the toxin, demonstrated by CDBAC at pH 3, is shared also by cetyl pyridinium chloride and cetyl trimethyl ammonium bromide. When the type A toxin was allowed to interact with CDBAC at pH 1, an entirely analogous result was obtained (*i.e.*, only partial inactivation of the toxin at mass ratios of CDBAC to toxin as high as 51). This should be contrasted with the complete inactivation of the type A toxin by the same surface active agent at pH 7.2 obtained at a mass ratio of CDBAC to toxin of 0.51.

TABLE 5

INTERACTION OF TYPE B BOTULINUM TOXIN ON THE ACID SIDE (pH 3) OF THE ISOELECTRIC POINT AND CDBAC

Mass ratio SAA/toxin*	*Toxicity (MLD × 10^3) per ml. of the mixture*
Control (no SAA)	1,000
0.16	1,000
0.24	1,000
0.48	1,000
0.72	1,000
0.96	1,000
1.2	1,000
1.6	1,000
2.0	316
5.0	316
10.0	316
20.0	316
50.0	316

* SAA = surface active agent.

The nonionic surface active agent, Triton A-20, did not alter the toxicity of the botulinum toxins on either side of the protein isoelectric points or at any mass ratio of the reactants. Other nonionic surface active agents gave similar results even though they differed from Triton A-20 in the nature of their hydrophobic residues, the nature and molar ratio of their alkylene oxide content, or the degree of their polymerization. These results are of some interest in that most of these nonionic compounds do not share the low toxicity and meager hemolytic activity displayed by Triton A-20.[17]

Studies on the inactivation of partially purified preparations of influenza and psittacosis viruses by surface active agents again confirm the importance of variables[22] such as the ionic nature of the agent, the pH of the solution, and the mass ratio of the reactants in determining precipitation, redispersion, and inactivation.

Discussion

The titration method which has been described for following the interaction of proteins and surface active agents by changes in the optical density of their mixtures is rapid, sensitive, and semiquantitative. The changes in optical density observed in these protein solutions during titration by surface active agents are proportional to the aggregation of the protein-surface active agent complex and may be tabulated as a function of either the volume of surface active agent solution used or the surface active agent to protein mass ratio. Since the transmittance of these mixtures would be affected by changes in both the size and number of aggregates formed, and no means of evaluating the relative contribution of each of these factors was available, use of this method was limited to semiquantitative orientation experiments. Within these limitations, however, the method is valuable for studying such problems as the influence of pH and the mass ratio of the reactants on the precipitation and redispersion of proteins by surface active agents. Methods, similar in principle, have recently been described for the titrimetric analysis of ionic surface active agents[23] and for the estimation of urinary protein concentration.[24] In this latter application, the appearance of a definite turbidity is estimated visually, in contrast to the photometric measurement of the progressive change in turbidity during titration used by Lambert[23] and in the present report.

The pH of the system has been seen to exert a marked effect upon the efficiency of a given ionic surface active agent as an inactivator of botulinum toxin. Cationic compounds have exhibited maximum efficiency in alkaline solutions, while anionic compounds have been most efficient in acid solutions. In both these situations, the surface active compound and the toxin bear opposite net electrostatic charges. These facts have important practical implications in the employment of ionic surface active agents for detoxification purposes in laboratory handling of powerful bacterial toxins. Studies of the detoxification of crude but nevertheless highly potent preparations of both botulinum and tetanus toxins by commercial grades of surface active agents fully confirm this conclusion.[17]

Proteins have been demonstrated to be precipitable upon interaction with definite mass ratios of surface active agents of appropriate chain length when the reactants bear net electrostatic charges which are opposite in sign. It is of interest to note that there exist both qualitative and quantitative analogies in the precipitation experiments carried out on the acid side of the protein isoelectric point with the anionic surface active agent and on the alkaline side of the protein isoelectric point with the cationic surface active agent. It has been suggested by Putnam[2] on the basis of studies with the anionic compound, dodecyl sulfate, that not only are the complexes formed between horse serum albumin and this surface active agent stoichiometric in character, but the molar ratio of the reactants may be determined by the number of positively ionized groups of the protein. Our results confirm this view, and we find that similar factors are of importance with relation to the binding of cationic surface active agents (keeping in mind, of course, the difference in sign of the electrostatic charge). While we do not possess the requisite physical data to define accurately the ratio of reactants necessary for formation of specific complexes, it is of interest to note the relationship between the total anion or cation binding capacity of proteins we have studied and the surface active agent to protein mass ratio found in our quantitative precipitation experiments. Utilizing the analytical data of Brand (cited by Edsall[25]), the calculated ratio of Lorol to crystalline bovine plasma albumin is 0.47 and the observed ratios for maximal precipitation are 0.22 to 0.41. For the same protein and CDBAC, the calculated ratio is 0.58 and the observed ratios are 0.34 to 0.62. On the basis of analytical data of Buehler, *et al.*,[26] the calculated ratio of Lorol to type A botulinum toxin is 0.26, and values of 0.12 to 0.36 have been found experimentally. Analogous calculations have been made for crystalline lysozyme, pepsin, and egg albumin, with similar evidence of an ability to predict the mass ratio of surface active agent to protein necessary for maximal precipitation from the total anion or cation binding capacity of the protein.[17]

These results are consonant with the hypothesis of strong polar groups in surface active agents being requisite to interaction with proteins. The lack of interaction of nonionic surface active agents and proteins has important consequences, since it allows their coexistence in solution with maintenance of altered physical characteristics, such as lowering of surface tension, solubilization, *etc.*, but divorces them from such biologically deleterious effects as denaturation and inactivation.

Summary

In a study of the interaction of crystalline bovine plasma albumin and purified preparations of types A and B botulinum toxin with surface active agents, precipitation of these proteins has been observed when the surface active compound is of an appropriate chain length and the two reactants bear opposite net electrostatic charges and are present in appropriate mass ratios. These interactions may result in inactivation of the toxin, with the mass ratio of the reactants and the pH of the solution again as important variables. The nonionic surface active agent did not cause precipitation or inactivation of the toxin on either side of the protein isoelectric point.

These results, utilizing relatively highly purified and isolated reactants, demonstrate many analogies to the effects of surface active agents on more complex biological systems such as bacteria.

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CONDUCTOMETRIC STUDIES OF BACTERICIDAL MECHANISMS

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Release of solutes by bacteria upon treatment with surface-active agents has been demonstrated by Hotchkiss.* Chemical analyses of washings of bacterial suspensions containing added agents were used. The present work presents a new technique in the study of the effect of cationic surface-active substances upon bacteria. Release of solutes is followed as it occurs by measurement of the electrical conductance of suspensions of bacteria upon treatment with surface-active materials.

Experimental. Conductances were measured in a cell with unplatinized platinum electrodes. A drain tube connected the cell with a flask so that a solution could be transferred by application of air pressure from one to the other without opening the system. A buret containing the bactericide solution was set up to drain into the flask. The cell, mixing flask, and buret were continuously surrounded by constant-temperature water at 30.0°C.

In operation, a preliminary measurement was made of the bacterial suspension alone after allowing it to come to thermal equilibrium. Then the suspension was run back into the mixing flask, a measured volume of bactericide solution was added, and mixing was effected by transferring the mixture from flask to cell several times.

Bacteria for the suspensions were obtained by collecting the test organisms after two-day growth on tryptone-glucose-extract agar at 37°C. The cells were washed three times with, and suspended in, autoclaved conductivity water.

Results and Discussion. In FIGURE 1, equivalent conductivity is plotted against the square root of normality. Equivalent conductivity, which is specific conductance divided by concentration, gives a measure of the conductance per molecule. The broken line represents the conductance curve for pure dodecylammonium chloride, the break in the curve at the concentration represented by the value 0.12 corresponding to the critical micelle concentration. The upper curve shows what can be termed the apparent equivalent conductivity of dodecylammonium chloride in the presence of living bacteria. To a suspension containing 55 billion bacteria (*Staphylococcus aureus*) per cc., a concentrated solution of the amine salt was added, producing the concentrations represented by the points on the curve. In each case, the conductance was determined after a 15-minute exposure. Additional portions of amine-salt solution were added to the same bacterial suspension. Therefore, the effect at each point includes the cumulative effects of previous concentrations and exposures. The original suspension of live bacteria had an appreciable conductance, which was deducted in plotting the curve. The significant feature of this curve is the pronounced

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* Hotchkiss, R. D., Ann. N. Y. Acad. Sci. 46 (6): 479, 1946.

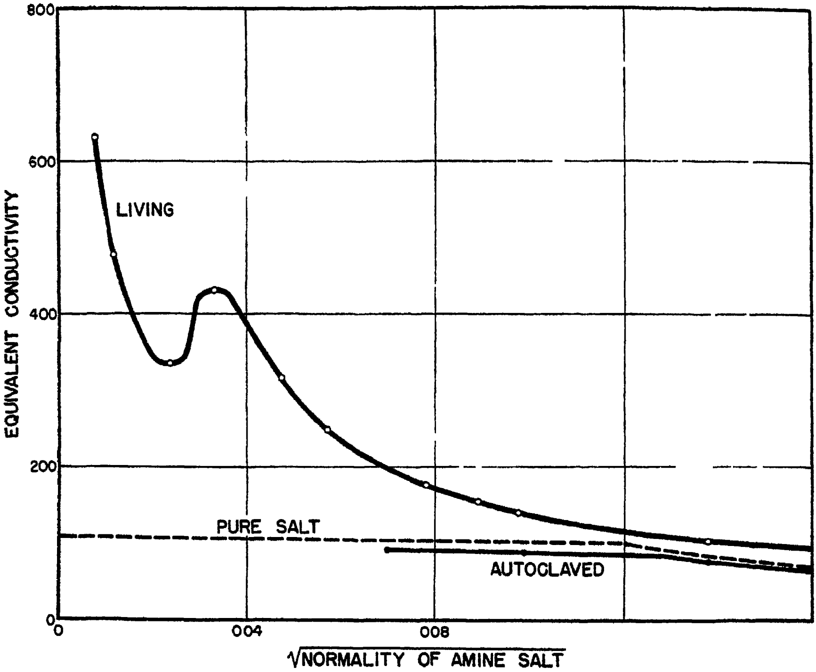

FIGURE 1. Equivalent conductivity of dodecylammonium chloride alone and in the presence of living and dead *S. aureus*. Conductances due to the original bacterial suspensions have been subtracted.

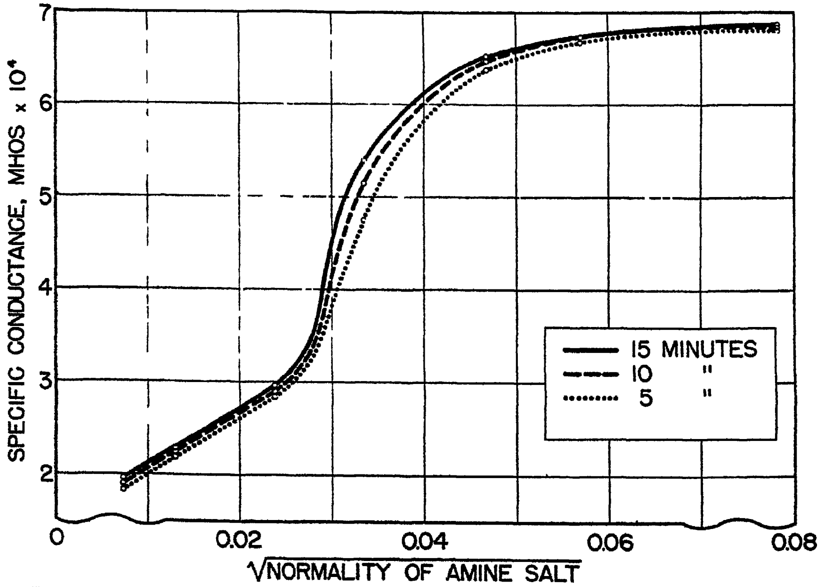

FIGURE 2. Specific conductance attributable to *S. aureus* in the presence of dodecylammonium chloride.

hump. This hump represents an abrupt increase in the conductance of the mixture, which can be accounted for only by the release of ionic material by the organisms. Almost complete killing of the organisms occurs at the point represented by the peak. Plotting this total conductance as equivalent conductivity of amine salt gives fictitious values but serves to emphasize the effect.

The lower solid line represents a similar experiment using dead bacteria. No evidence of a hump was obtained, either in the curve shown or in other experiments extending into regions of lower amine-salt concentrations.

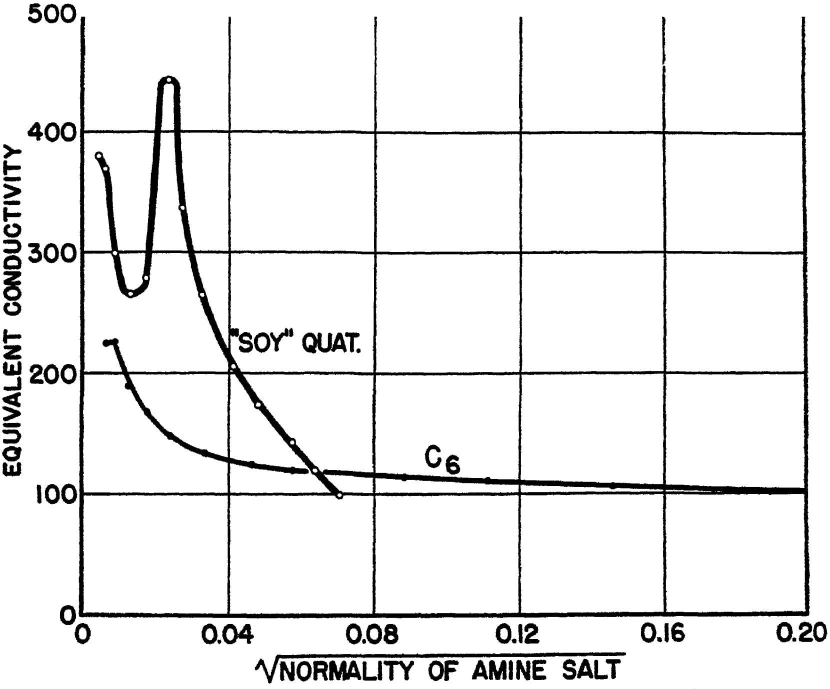

FIGURE 3. Equivalent conductivities of trimethyl-"soy"-ammonium chloride and hexylammonium chloride in the presence of living *S. aureus*. Conductances due to the original bacterial suspensions have been subtracted.

The same effect is illustrated in a slightly different manner in FIGURE 2. In this case, *specific* conductance is plotted against the square root of normality. The values for the conductance of the amine salt have been deducted, and the curves, therefore, indicate only the conductance attributable to the bacteria. The three points at a given concentration show the conductances 5, 10, and 15 minutes after addition of the amine salt. These curves indicate that in concentrations below the break very little change occurs with prolonged exposure. At concentrations corresponding to the hump in FIGURE 1, the change in conductance with time is very much more pronounced, as evidenced by the spreading of the curves.

A plot of equivalent conductivity in the presence of live bacteria (*S. aureus*) is presented in FIGURE 3. The conductance of the bacterial suspension has been deducted. In this case, two different salts have been used. The upper curve represents the effect produced by trimethyl-"soy"-ammonium chloride, a quaternary ammonium salt containing the higher alkyl chains found in soybean oil. Here, again, we find the pronounced hump. The lower curve shows the effect of hexylammonium chloride—no hump is produced. It is well known that the quaternary ammonium salt is a powerful bactericide and that hexylammonium chloride has little or no bactericidal activity.

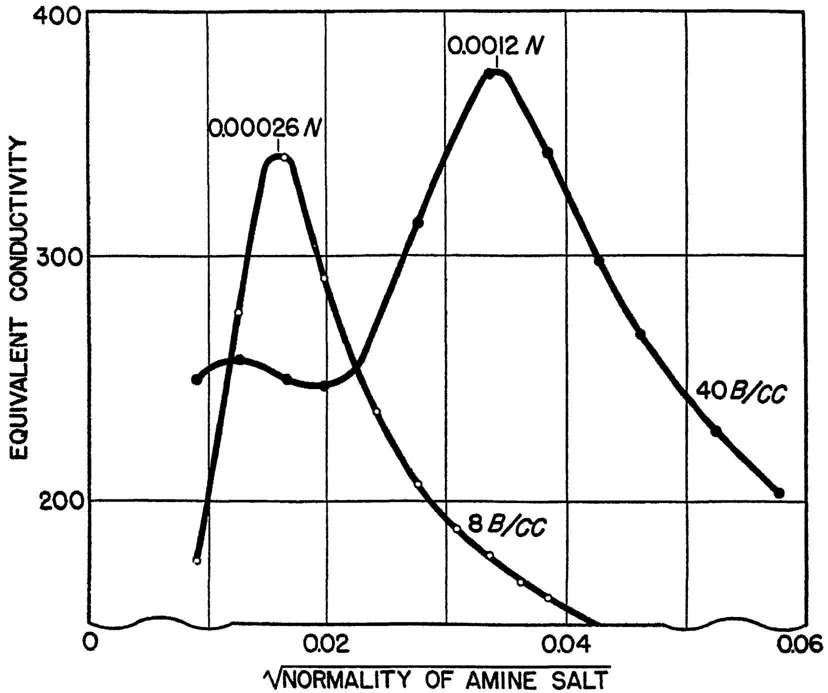

FIGURE 4. Effect of bacterial concentration on the equivalent conductivity of dodecylammonium chloride in the presence of living *S. aureus*. Conductances due to the original bacterial suspensions have been subtracted.

The effect of varying the bacterial concentration is demonstrated in FIGURE 4. Here, again, equivalent conductivity is plotted against the square root of normality. The conductance of the original bacterial suspensions (*S. aureus*) has been deducted. The curve at the left shows the effect produced when a suspension containing 8 billion organisms per cc. is treated with dodecylammonium chloride. The curve at the right represents the effect with a suspension containing 40 billion organisms per cc. The normality of the amine salt at the peak is shown in each case. It will be noted that the ratio of the concentration of the organisms is 1 to 5, and that the ratio of the normality at the peaks is also approximately 1 to 5. This may be coincidental, but calculations based upon a bacterium diameter of 1 micron and a minimum parking area of 20 square Angströms per molecule for the amine salt indicate that at 0.001 *N* sufficient salt is available to cover completely 40 billion bacteria per cc. No evidence is available that complete adsorption of the amine salt has occurred.

FIGURE 5 represents the data from one experiment. It is felt that considerably more work should be done before too much significance is attached to the quantitative aspects of these observations. The curves show in a different way the effect of time and chain length on the conductance of bacterial suspensions (*S. aureus*) in the presence of amine salts. Specific conductance is plotted against time. The conductances of the amine salts and the original bacterial suspension have been subtracted, so the effect shown is to be attributed only to the conducting material released by the bacteria. In each case, the concentration of amine salt was .001 *N* and that of bacteria, originally 6 billion per cc. Conductance measurements were made at 5-minute intervals. The numbers on the curves show the bacterial counts at the indicated times.

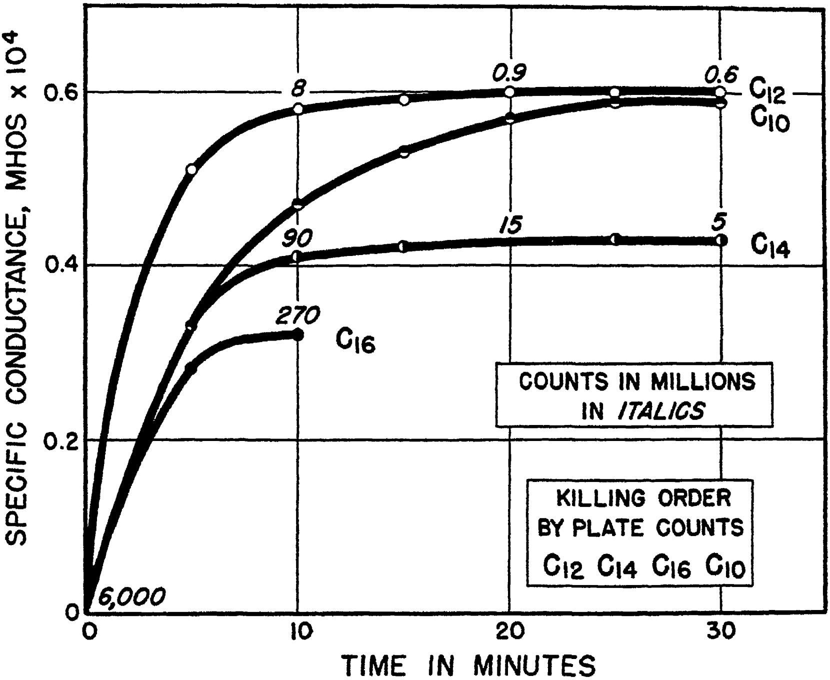

FIGURE 5. Effect of time on the specific conductance of material released by *S. aureus* in the presence of 0.001 *N* solutions of alkylammonium chlorides. Conductances due to the alkylammonium salts and the original bacterial suspensions have been subtracted.

Several interesting features in this set of curves should be pointed out.

The order of the curves for the dodecyl, tetradecyl, and hexadecyl compounds is the same as the order of killing, as indicated both by counts made at the time of the experiment and by the usual method of determining activity by plate counts. The curve for decylammonium chloride appears to be out of order. In this case, exact counts were not obtained, since the killing was of a much higher order than expected. Less than 10 million organisms per cc. survived. It is felt that the shape of this curve, which indicates a more gradual effect, is significant, as is the leveling off shown by the curves for the other substances.

In speculating as to the meaning of this set of curves, three tendencies on the part of the amine-salt molecule should be considered: the tendencies toward micelle formation and toward adsorption by the organisms, both opposing the tendency toward remaining in solution. The bactericidal behavior of a given salt will be a function of these tendencies, which are related to the length of the carbon chain and to the nature of the polar group. Decylammonium chloride has relatively little tendency toward micelle formation and is very soluble. This is reflected in a more gradual killing action. Hexadecylammonium chloride, on the other hand, has a low solubility and a high tendency toward micelle formation, with the result, perhaps, that fewer molecules are available for adsorption on the organism. Little is known of the actual forces which bring about attachment of the amine salt to the organism, but this attachment probably is through the polar or hydrophilic group. Properties of this group are, of course, influenced by the carbon chain.

It has usually been assumed that the release of solutes as a result of the treatment of bacteria with surface-active materials is due to adsorption on the bacterial membrane resulting in damage to the membrane and subsequent loss of cell contents. Probably, the effects presented in this work can be accounted for in the same manner. The possibility should not be overlooked, however, that the release of conducting material is evidence of a defensive mechanism on the part of the organism. The fact that this effect is noted at concentrations very much below that required for killing seems to support such a hypothesis.

In a study of bactericidal properties, variation in the resistance of individual organisms plays an important part. Price* has presented a clear exposition of this point. Interpretation of the effects shown by FIGURE 5 is difficult without a more complete knowledge of this variation in bacterial resistance. It is probable that the conductometric method is one approach to the study of this very problem.

That the conducting material released by organisms upon treatment with cationic surface-active materials is not the product of a simple replacement reaction is definitely indicated. To account for the observed increase in conductance, the liberation of an ion having a much higher equivalent conductance than sodium, potassium, or ammonium would be necessary.

In an attempt to throw some light on the question of cell-wall rupture, studies were made with the electron microscope.[*] An electron micrograph of *S. aureus* organisms killed by treatment with a solution of dodecylammonium chloride, when compared with a similar picture of living organisms, showed morphological change but no evidence of rupture of the cell walls. This, of course, does not preclude the possibility of damage to the cell walls without actual rupture.[†]

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* PRICE, P. B., Ann. N. Y. Acad. Sci. 53 (1): 76. 1950.

All of the work thus far reported has employed *S. aureus* as the test organism. Other experiments in which *Escherichia coli* and *Bacillus subtilus* were used demonstrated similar effects. One experiment in which phenol was employed as the bactericidal agent also showed an abrupt increase in conductance at the killing concentration.

Conclusion. The conductometric method affords a valuable technique for use in the study of the mechanism of bactericidal activity. It offers a means for the immediate detection of an effect of surface-active agents upon bacteria, even at concentrations far below that required for complete killing. It is not unlikely that the technique can be made the basis for a quantitative estimation of bactericidal activity. Such an application will require much more extensive investigation.

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[*] This study was performed by Mr. C. E. Tufts of the Armour Research Foundation.

[†] The hemolytic effect of surface-active agents is well known. It was suggested by Dr. H. L. Davis that an effect on conductance similar to that observed with bacteria might also occur with red blood cells. An experiment was performed after the presentation of this paper to examine this possibility. A suspension containing 6 billion rabbit red cells per cc. of isotonic saline was treated with dodecylammonium chloride solution. At the concentration at which hemolysis became very pronounced (0.001 N), an abrupt *decrease* in equivalent conductivity occurred. This indicates the release of relatively nonconducting material plus the removal of conducting substances, probably through adsorption of amine salt by proteinaceous material.

A NEW AND DIRECT APPROACH TO THE EVALUATION OF THE GERMICIDAL EFFICIENCY OF SEMISOLID PHARMACEUTICALS

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In recent years, there has been considerable progress in the development of new and improved germicides, and many modifications and innovations in the test methods for germicides have been proposed. There has also been progress in the compounding of ointment bases. Departures from the greasy type now include creams and water-miscible bases. Comparatively little, however, has been reported upon the test methods for evaluation of germicidal ointments. When one is confronted with a product purported to have germicidal qualities, the usual procedure is to employ the Himebaugh cup-plate method (possibly subculturing a portion of agar from the clear zone after 24 hours) or the filter paper method. Both of these tests are set forth in Circular 198, which was published by the USDA in 1931.[1]

Although introduced some 18 years ago, the filter paper technics (wet and dry) are not widely used. The agar cup plate method is resorted to as the alternative and because it is simple. It is the purpose of this paper to discuss some of the limitations of the agar plate test method, and to present a new and direct approach to the evaluation of the germicidal efficiency of semisolid pharmaceuticals (ointments, creams, jellies, *etc.*), with emphasis on those containing the quaternary ammonium germicides.

Limitations of Agar Plate Test Method. The agar plate test has a number of limitations and objections to its use.

(1) The method is limited to test organisms that will give suitable growth on agar. Many organisms present at the site of application of ointments and creams (TABLE 1) are never employed in the agar plate method because they do not grow satisfactorily on the regular agar medium used. The conditions of the test (temperature, oxygen, medium, pH, *etc.*) are determined by the optimum conditions for the test organism, rather than by the conditions of use for the product under test. Thus, the conditions of test using *Staphylococcus* as the test organism will not be the same as if a clostridium, mycobacterium, or a pathogenic fungus is used. Clinically, the products would be used under the same conditions, regardless of the type of organism.

(2) No provision is made for the evaluation of more rapid action. Subcultures to determine germicidal action, if any, are made after 18–24 hours incubation, after the zone of inhibition has appeared. The data in TABLE 2 show that the agar plate test alone is not a good criterion of germicidal action. Sample 67 shows a good zone size, one larger than might be expected from the quaternary alone. By the germicidal test, the germicide is actually shown to be inactivated. The addition of the antibiotic resulted in a definitely larger zone, but did not improve the germicidal activity. Further study of the product ingredients showed that the tegacid used inactivated

the quaternary ammonium salt. By the agar plate test this incompatibility would not have been found. With regard to the effect of germicide neutralization on zone size and inactivation of germicidal activity, the results with Tamol-N are of interest (TABLE 3). Tamol-N has been proposed as an inactivator of quaternary ammonium compounds by Goetchius.[3] These

TABLE 1
MICROORGANISMS WHICH MAY BE PRESENT AT SITE OF APPLICATION OF OINTMENTS, CREAMS, JELLIES, *etc.*

	Pus	*Wounds*	*Skin*	*Eyes*
Staphylococci (sp)	X	X	X	X
Pneumococci		X	X	X
Streptococci (sp)	X	X	X	X
B. subtilis	X	X	X	X
B. anthracis	X		X	X
Clostridia (sp)	X	X		
Corynebacteria (sp)		X	X	X
Mycobacteria (sp)	X		X	
Erysipelothrix			X	
Actinomyces (sp)			X	X
Neisseria (sp)			X	X
Pseudomonas aeruginosa	X	X	X	X
Escherichia coli	X	X	X	
Proteus vulgaris	X	X	X	
Borrelia vincentii		X	X	
Non-bacterial forms (fungi, *etc.*)			X	

TABLE 2
INGREDIENT INCOMPATIBILITY AS MEASURED BY ZONE SIZE AND GERMICIDAL ACTIVITY

Sample	*Composition*			*Himebaugh cup plate zone size*	*Survival of* S. aureus *by CKT germicidal test** *(in min.)*						
	germicide	*antibiotic*	*vehicle*		*½*	*1*	*1½*	*2*	*3*	*4*	*5*
Control	1:500	0	water	3 mm.	−	−	−	−	−	−	−
−67	1:500	0	soft cream†	3 mm.	+	+	+	+	+	+	+
−61	1:500	1:1000	soft cream	7 mm.	+	+	+	+	+	+	+
Control	1:500	0	water + calamine	—	−	−	−	−	−	−	−
Control	1:500	0	water + tegacid	—	+	+	+	+	+	+	+

* Critical Killing Time test at 37°C. and Letheen[2] broth, upon a 1:8 dilution of sample in water. (−) indicates killings; (+) indicates survival.

† The soft crean contains calamine, tegacid, benzyl alcohol, aromatic oils, and other ingredients. Germicide-quaternary ammonium salt.

data show that 4 per cent Tamol-N is not germicidal or bacteriostatic. Mixtures with Ceepryn* (cetyl pyridinium chloride) are germicidally inactive under the test conditions but produce an enhanced zone size in the agar plate test.

A comparison between the filter paper technic results and the "cidal" zones reported by Hart and Huyck[4] are of significance (TABLE 4). The results show a lack of correlation. In fact, a reduction in the Hyamine 10-X content of the ointments may have no influence on the zone size (*), may decrease the zone size (**), or may increase the zone size (***).

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* Ceepryn—trademark of The Wm. S. Merrell Company.

(3) A third objection to the agar plate method is that germicides of certain types, or of large molecular size, may not diffuse through the agar. Hoogerheide,[5] in his work on cetyl trimethyl ammonium bromide, reported on this lack of diffusion through agar (TABLE 5). The C-12 homologue produced the largest zone in the agar plate test, but it was actually less germicidal than the C-14, C-16, and C-18 compounds. He indicates that the smaller

TABLE 3

RELATION OF GERMICIDE NEUTRALIZATION TO ZONE SIZE

Ceepryn	*Tamol-N*	*Agar plate zone size** S. aureus	*Survival of* S. aureus *by CKT germicidal test*† (*in min.*)						
			½	*1*	*1½*	*2*	*3*	*4*	*5*
0	4%	no zone	+	+	+	+	+	+	+
1:50	0	2.5 mm.	−	−	−	−	−	−	−
1:50	1:50	4.37 mm.	+	+	+	+	+	+	+
1:200	0	1.87 mm.	−	−	−	−	−	−	−
1:200	1:200	2.25 mm.	+	+	+	+	+	+	+

* Using S & S filter paper discs, average of four zones.
† Critical Killing Time test at 37°C. and Letheen broth. (−) indicates killing; (+) indicates survival.

TABLE 4

CORRELATION OF GERMICIDE TEST (FILTER PAPER TECHNIC) WITH AGAR PLATE TEST†

Ointment base	*Germicide Hyamine 10-X*	*Filter paper technic*			*Agar cup plate. 20°C.* S. aureus	
		5	*10*	*15*	*diffusion*	*cidal zone*
		Minutes‡				
Modified hydrophilic	2%	−	−	−	8 mm.	3 mm
	1%	−	−	−	18	3
	0.1%	+	+	+	1.5	1.5
White ointment	2%	−	−	−	3	3
	1%	+	+	+	2	2
	0.1%	+	+	+	2	0
Modified hydrophilic petrolatum	2%	−	−	−	6	2
	1%	−	−	−	6	3 }*
	0.1%	−	−	−	3	3
Phenol ointment base	2%	−	−	−	5	3
	1%	+	+	+	2	2 }**
	0.1%	+	+	+	0	0
Burn ointment base PRB	2%	−	−	−	7	7
	1%	−	−	−	12	0 }***
	0.1%	+	+	+	4	4

† Data from Hart and Huvck.[4]
‡ (−) indicates killing; (+) indicates survival.

C-12 molecule is able to diffuse more freely through agar than are its higher homologues.

The effect of agar on the quaternary ammonium salts has been reported by Quisno, Gibby, and Foter.[6] Their findings indicate a physical adsorption which causes a reduction in germicidal potency of these germicides. They reported that agar cup plate tests are inappropriate for the evaluation of preparations containing quaternary ammonium salts. Sherwood,[7] also, has observed that agar decreased the bactericidal activity of quaternary ammonium salts. The effect was associated directly with the agar itself.

(4) The fourth objection is that agar in solution forms a gelose or carbohydrate sol and does not present a medium which simulates skin conditions.

The Committee on Cosmetics of the A.M.A. has described[8] skin as a continuous, relatively smooth layer of dead, flattened keratinized cells. The pH varies from 3.5–7.0. Evaporation of sweat leaves behind salt, urea, and other substances. Sebum covers all areas except the palms and soles. Fat exists as a more or less uniform layer. The cells contain proteins, lipids, and water. Skin may differ in texture, amount of hair, pigmentation, fatty secretions, and residues from perspiration.

TABLE 5

CORRELATION OF MOLECULAR SIZE WITH ANTIBACTERIAL ACTIVITY*

Quaternary ammonium compound	*CKD*† S. aureus *37°C.*	*Glass cylinder plate.* S. aureus	
		complete zone	*partial zone*
C-9-trimethyl ammonium bromide	1:1500	0.5 mm.	4.0 mm.
C-10-trimethyl ammonium bromide	1:7000	1.5	6.0
C-12-trimethyl ammonium bromide	1:70,000	5.5	5.0
C-14-trimethyl ammonium bromide	1:340.000	4.0	1.0
C-16-trimethyl ammonium bromide	1:400,000	0.5	1.0
C-18-trimethyl ammonium bromide	1:400,000	0	0.5

* Data from Hoogerheide.[5]

† CKD = Critical Killing Dilution—that which kills in 10 minutes but not in 5 minutes.

The recent evaluation, by Brown and his associates,[9] of glycerite of hydrogen peroxide with 32 commercial solutions as skin antiseptics, solely by means of the agar plate test, would not appear valid from the data and literature cited.

(5) The next objection is the lack of clear-cut correlation between zone sizes and clinical results, especially in the field of antifungal preparations. A step in the right direction was made by Meyers[10] and his associates in their study of *in vivo* absorption of potassium iodide in rats from various ointment bases and the agar plate zones produced by these ointment bases. The ointment base plus 10 per cent lanolin was the best with regard to *in vivo* absorption of the potassium iodide through the skin of rats, but not by the agar plate tests.

Hopkins and his coworkers[11] reported on the clinical performance in dermatophytosis of organo-mercurials at 29 per cent cure and an undecylenate ointment at 79 per cent cure. By the agar plate test, just the reverse would be observed, since the organo-mercurials are known to give larger zones than sodium undecylenate.

With regard to improvement of the agar plate method itself, much of the recent literature has been devoted to the investigation and standardization of minor points in the technic. A few, such as Tobie and Ayres,[12] have suggested that this test procedure has led us astray. If one depended solely upon the agar cup plate method for screening new germicides and antibiotics, the quaternary ammonium salts and tyrothricin might not have been recognized, since little to no zones of inhibition are obtained by the agar plate test.

Rose and Miller[13] reported studies on the technic of the agar plate method with the hope of obtaining more consistent results. They studied 13 factors or conditions in the test. After studying all these factors and conditions, irrational results were obtained. Five per cent phenol, which is definitely germicidal, by the agar plate test, displayed less than half the activity of 0.1 per cent crystal violet. It should be noted that these were solutions and not ointments.

TABLE 6

CORRELATION OF GERMICIDAL ACTIVITY OF SULFA OINTMENTS WITH ZONE SIZE*

Ointment	*Self-sterilizing test* S. aureus (*time in hours*)†								*Zone size-mm.* S. aureus
	1	*2*	*4*	*6*	*8*	*18*	*24*	*48*	
Sulfathiazole ointment	+	+	+	+	+	+	+	+	0
Sulfathiazole + 1% benzoyl peroxide	+	−	−	−	−	−	−	−	0
Sulfathiazole + 1% urea peroxide	+	−	−	−	−	−	−	−	15–17
Sulfathiazole + 1% zinc peroxide	+	+	+	+	+	+	+	+	4
Sulfadiazine ointment	+	+	+	+	+	+	+	+	0
Sulfadiazine + 1% benzoyl peroxide	+	−	−	−	−	−	−	−	0
Sulfadiazine + 1% urea peroxide	+	+	−	−	−	−	−	−	10
Sulfadiazine + 1% zinc peroxide	+	+	+	+	+	+	+	+	0
Sulfanilamide ointment	+	+	+	+	+	+	+	+	0
Sulfanilamide + 1% benzoyl peroxide	+	−	−	−	−	−	−	−	0
Sulfanilamide + 1% urea peroxide	+	+	−	−	−	−	−	−	15
Sulfanilamide + 1% zinc peroxide	+	+	+	+	+	+	+	+	1

* Data from Fisher, Accousti, and Thompson [17]

† (−) indicates killing; (+) indicates survival.

U.S.P. ammoniated mercury ointment, which produces a 2 mm. zone, had been suggested as a standard by Bryan,[14] who studied 80 products in official compendia. On the other hand, Prout and Strickland[15] studied fatty and non-fatty ointment bases for their antiseptic activity by the agar plate test. Only 4 out of every 10 official products were found active, even though these products are generally indicated for use in conditions demanding antiseptic activity. Gershenfeld and Zepeda[16] have also published on ammoniated mercury ointment offered as the standard. These studies center on the proper control ointment, the method of applying the ointment to the agar, and the method of measuring. Regardless of the amount of ointment used, the method of application, or the area covered by the ointment, the zone size was about the same.

In 1943, the first approach to a more direct method of evaluating the antiseptic properties of ointments was taken by Fisher, Accousti, and Thompson[17] in their evaluation of sulfa ointments. In the method used by these workers, 1.0 cc. of the test culture was added to 4 grams of ointment in a sterile jar and the two mixed. *Streptococcus hemolyticus*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium tetani*, and *Clostridium perfringens* were employed. At stated time intervals, subcultures were made with a 4 mm. loop to either nutrient or blood broth. This is really a germicidal test method, though it was not introduced as such. In addition, agar plate tests were made. The results are reproduced in TABLE 6. The germicidal activity on addition of 1 per cent benzoyl peroxide to the sulfonamide base ointments is not reflected in the zone size.

Prusak and Mattocks[18] have recently published on the compatibility of various ointment bases with cetyl trimethyl ammonium bromide. In addition to a serial dilution bacteriostatic test, they employed a direct germicidal evaluation. This was a more valid approach, but the methods are open to criticism in that they introduced more water in the form of broth than would ordinarily be encountered under conditions of use.

The trend of study of the agar plate test method was reviewed by Reddish[19] in 1938, and, in a "Bulletin of the National Formulary Committee"[20] in 1947, the agar plate test was discussed and interpreted in detail. The interpretations of the agar plate test in these publications are of interest here.

(1) "This test demonstrates not only the presence or absence of an antiseptic ingredient in the ointment, but also determines the relative amount of the active ingredient." In TABLE 6, the addition of benzoyl peroxide (1 per cent) to sulfa ointments resulted in germicidal preparations but gave no zones by the agar plate method. Hart and Huyck's[4] data (TABLE 4) on the addition of various concentrations of Hyamine 10-X to ointments showed no direct correlation between the amount of active ingredient and zone size.

(2) "The size of the zone of inhibition given by this method is important and has a direct bearing on the clinical effectiveness of the ointment being tested." The work of Hopkins[16] on the clinical performance of organomercurials and an undecylenate ointment indicates the absence of correlation between zone size and clinical effectiveness. This is also true of quaternary ammonium germicides and tyrothricin containing preparations.

(3) "U.S.P. ammoniated mercury ointment is standard and, since it gives a 2 mm. zone, then antiseptic ointments must give a minimum zone of 2 mm. if they are to be considered effective antiseptic ointments. . . ." In the case of the homologues of cetyl trimethyl ammonium bromide, the most germicidal compounds gave zones of 1 mm. or less (TABLE 5). An ointment which meets this standard of 2 mm. against *S. aureus* might not meet this standard against other test organisms.

(4) It is recommended that Sabouraud's agar be employed for the evaluation of antifungal ointments, and that F.D.A. serum agar be employed for antibacterial ointments. It can be questioned whether Sabouraud's agar and F.D.A. serum agar simulate the skin surface, since these media differ in composition (pH, nutrients).

(5) For liquids that remain in long contact, such as wet dressings, it is indicated that the same test method be employed as for ointments. An antiseptic wet dressing must produce a 7 mm. zone, yet an antiseptic ointment need only produce a 2 mm. zone. It is known that many clinically effective wet dressings, because of their chemical nature, may not produce such zones.

By the studies cited and discussed, an attempt has been made to establish the limitations of the agar plate test method as a means of evaluating the *in vitro* germicidal properties of semisolid pharmaceuticals. Although this method has been in use for almost 20 years, there is still a lack of agreement between this *in vitro* test and clinical performance.

Experimental. In the evaluation of liquid germicides, the method most generally employed is one of two, both derived from the "phenol coefficient" technic. The one method holds the time constant. It determines the greatest dilution that will kill in 10 minutes, but not in 5; hence it may be designated a "Critical Killing Dilution" test. The other method holds the dilution constant (generally at actual use concentration) and determines the time required for that dilution to effect killing. This would be a "Critical Killing Time" test. Both methods use the usual 5.0 cc. of test material, to which is added 0.5 cc. of culture. These methods are versatile in that different temperatures, subculture methods and media, test organisms, *etc.*, can be employed to suit the product and its use.

It was decided to investigate in a preliminary way whether such a direct and versatile procedure could be applied to other than liquid samples. A number of apparent objections have been explored. Others remain to be evaluated. The data thus far obtained are presented to stimulate thought toward a more suitable test method than the agar cup plate technic.

The semisolid germicidal test employed in these studies is carried out in the following manner. (1) A 10 gram sample is weighed into a 25 x 115 mm. test tube and placed in a 37°C. water bath. (2) 1.0 ml. of a standard culture of *S. aureus* (or other test organism) is added, and the mixture stirred with a sterile applicator for 1 minute. (3) The mixture is transferred to a 5 ml. syringe (glass tipped, without needle, and graduated in 0.2 ml.). The syringe is held in a clean short tube in a water bath. The temperature of the ointment mixture has been determined to be 34°C. when so manipulated. (4) At intervals of 5, 10, 20, 30, 60 minutes or longer, duplicate transfers of 0.4 ml. amounts in the syringe are planted into an appropriate subculture medium This medium should neutralize any carryover of germicide. Routinely, *S. aureus* is employed, with Letheen broth as the medium for quaternary ammonium compounds. (5) If the subculture tubes are cloudy from insoluble ingredients, a transfer is made after preliminary incubation, and both tubes are observed for growth. (6) Negative subculture tubes may be checked for conditions of bacteriostasis, simply by inoculation with a diluted test culture.

In our detailed study of this test method, two samples were employed. One was the petrolatum base type, containing 1:500 Ceepryn. The other was the cream type, containing 1:1000 Ceepryn. Conditions of the test studied were:

(1) *Subculture quantity.* Since the quantity of sample transferred by means of a 4 mm. loop varied tremendously, the syringe technic was introduced. In TABLE 7 appear the results of weighing experiments on both samples to show that the 0.4 ml. transfer is the most consistent. In addition, 0.1 ml. and 0.2 ml. transfer volumes were studied.

TABLE 7

VARIATION DUE TO METHOD OF SAMPLING

	Petrolatum base			*Cream base*		
	weight in grams	*deviation*	*% deviation*	*weight in grams*	*deviation*	*% deviation*
Standard loop	0.0912	+.0106	13.1	0.0490	−.0478	48.3
	0.0939	+.0132	16.3	0.0862	−.0106	10.8
	0.0805	−.0002	0.2	0.1275	+.0307	31.7
	0.0128	+.0221	27.4	0.0736	−.0233	24.0
	0.0736	−.0071	8.6	0.0908	−.0060	0.6
	0.0676	−.0131	16.2	0.1212	+.0244	25.2
	0.0720	−.0087	10.7	0.0869	−.0099	10.1
	0.0687	−.0120	14.8	0.1280	+.0312	32.2
	0.0683	−.0124	15.3	0.1179	+.0211	21.8
	0.0879	+.0072	8.9	0.0873	−.0095	9.8
Arith. mean:	0.0807		13.15%	0.0968		21.45%
0.1 cc. by syringe Arith. mean:	0.0862		3.79%	0.0881		12.18%
0.4 cc. by syringe	0.2942	−.0352	10.7	0.3674	+.0051	1.4
	0.3407	+.0113	3.4	0.3643	+.0020	0.5
	0.3236	−.0058	1.8	0.3449	−.0174	4.8
	0.3182	−.0112	3.4	0.3745	+.0122	3.4
	0.3306	+.0012	0.4	0.3715	+.0092	2.5
	0.3451	+.0157	4.8	0.3254	−.0369	10.2
	0.3103	−.0191	5.8	0.3860	+.0237	6.5
	0.3481	+.0187	5.7	0.3490	−.0133	3.7
	0.3227	−.0067	2.0	0.3577	−.0046	1.3
	0.3603	+.0309	9.4	0.3824	+.0201	5.5
Arith. mean:	0.3294		4.74%	0.3623		3.98%

TABLE 8

REPLICATE PLATE COUNTS SHOWING DISTRIBUTION OF CULTURE IN SAMPLE

Cream base sample	*Count per plate*	*Calculated per 0.4 cc.*	*Deviation from mean*	*% deviation*
#1	1106	22,120*	+3540	18.8%
#2	2036	40,720*	+21140	112.0%
#3	650	13,000	−5580	29.7%
#4	524	10,480	−8100	43.1%
#5	536	10,720	−7860	41.8%
#6	1126	22,520*	+3940	21.0%
#7	526	10,520	−8060	42.9%
Arithmetic mean:		18,580*		42.2%
Median:		13,000		

* These high values influence the arithmetic mean.

(2) *Distribution of culture through ointment.* This question was approached by the process of making replicate plate counts from the cream base mixture. Since the petrolatum base mixture was too rapidly germicidal, the cream base, which was known to effect killing in 20 to 30 minutes, was used. The routine test was carried out, except that, immediately after mixing in the culture, seven separate 0.4 ml. portions were planted into 20 ml. amounts of Letheen broth. These were shaken and 1 ml. portions were plate counted. The results are shown in TABLE 8. It will be noted that the erratic dispersion is on the high rather than the low side.

TABLE 9-A

RESULTS OF REPLICATE ASSAYS BY SEMISOLID GERMICIDE TEST—OPERATOR BCS

Date: 7–28–47 (time in minutes)						*Date: 7–29–47 (time in minutes)*					
Test	*5*	*10*	*20*	*30*	*60*	*Test*	*5*	*10*	*20*	*30*	*60*
1	+	+	−	−	−	1	+	+	−	−	−
2	+	+	+	−	−	2	+	+	−	−	−
3	+	+	+	−	−	3	+	+	−	−	−
4	+	+	−	−	−	4	+	+	−	−	−
5	+	+	−	−	−	5	+	+	−	−	−
6	+	+	−	−	−	6	+	+	−	−	−
7	+	+	+	−	−	7	+	+	−	−	−
8	+	+	+	+	−	8	+	+	−	−	−

(+) represents growth of *S. aureus*, or no killing; (−) represents killing of *S. aureus*.

TABLE 9-B

RESULTS OF REPLICATE ASSAYS BY SEMISOLID GERMICIDE TEST—OPERATOR VM

Date: 7–25–47 (time in minutes)						*Date: 7–28–47 (time in minutes)*					
Test	*5*	*10*	*20*	*30*	*60*	*Test*	*5*	*10*	*20*	*30*	*60*
1	+	+	+	−	−	1	+	+	−	−	−
2	+	+	−	−	−	2	+	+	−	−	−
3	+	+	−	−	−	3	+	+	−	−	−
4	+	+	+	−	−	4	+	+	−	−	−
5	+	+	+	−	−	5	+	+	−	−	−
6	+	+	−	−	−	6	+	+	−	−	−
7	+	+	+	−	−	7	+	+	−	−	−
8	+	+	−	−	−	8	+	+	−	−	−
9	+	+	−	−	−						
10	+	+	+	−	−						
11	+	+	+	−	−						

(+) represents growth of *S. aureus* or no killing; (−) represents killing of *S. aureus*.

(3) *Reproducibility of results.* Again working with the cream base, two different operators, two different samples, and different days were chosen for replicate assays. The results are presented in TABLES 9-A and 9-B. It will be noted that in all 35 tests killing was achieved in 60 minutes, in 34 there was killing in 30 minutes, in 25 in 20 minutes, and no killing in 5 and 10 minutes. It will be observed that there is fairly good reproducibility between results of different operators, different samples, and on different days.

(4) *Application to various products.* Eight different pharmaceutical semisolid preparations were tested in replicate by the semisolid germicidal test method described and also by the agar cup plate method. The results are shown in TABLE 10. Sample B is similar to A, except that it contains only one-tenth the amount of germicidal ingredient. By the agar plate test, there is no difference in activity, but there is in the germicidal test. In the case of sample D, the 9 mm. zone is shown to be due to other nongermicidal ingredients, since there is a complete loss of germicidal activity. Samples F and G have good germicidal activity and larger zones than might be expected. They are, no doubt, due to essential oils or other ingredients added to improve the pharmaceutical elegance of the product.

Still to be studied is the dispersion of the 0.4 ml. transfer sample in the subculture broth. This is mostly a problem in the case of petrolatum type bases. There remains to be decided just what constitutes a satisfactory activity. This would be a long-time correlation with clinical findings. And there is still some work to be done comparing results by this method and the wet and dry filter paper technics.

TABLE 10
CORRELATION OF SEMISOLID GERMICIDE TEST RESULTS WITH ZONE SIZE*

Type of semisolid	*"Quat" conc.*	*Tests run*	*Zone size mm*	*% of tests which show killing exposure time*							
				minutes					*hours*		
				5	*10*	*20*	*30*	*60*	*2*	*3*	*5*
(A) Ointment	0.2%	10	1 to 1½	100	100	100	100	100	100		
(B) Ointment	0.02%	6	1 to 1½	0	17	17	33	67			
(C) Ointment	0.2%	8	1 to 3	100	100	100	100	100	100		
(D) Cream	0.2%	5–23	9	0	0	0	0	0	0	0	0
(E) Emulsion (liquid)	0.2%	15	1 to 2	60	67	60	80	100			
(F) Jelly	0.1%	5	2 to 5	100	100	100	100	100	100		
(G) Ointment (soluble)	0.2%	5	3 to 5	100	100	100	100	100	100		
(H) Cream	0.1%	35	1	0	0	71	97	100	100		

* Test Culture *S. aureus*

Summary

The limitations and inadequacies of the agar plate test method have been indicated and discussed. It has been established that this test method is not a satisfactory one for the evaluation of the germicidal action of semisolid pharmaceuticals. It should not be employed to compare the germicidal activity of compounds of dissimilar chemical nature or molecular size.

No prediction of the clinical effectiveness of preparations should be made on the basis of the agar plate zone sizes.

The use of the agar plate test as the sole screening method will result in questionable data, for by this method many compounds and formulations which are actually highly germicidal are shown to have only slight activity.

A direct method for the evaluation of the germicidal efficiency of semisolid pharmaceutical preparations has been described, employing a "Killing Time" type of test. Data have been presented to show degrees of variation in (1) dispersion of culture through the sample, (2) methods of sampling, (3) replicate tests of one or more assayists, and (4) the applicability of the method to the germicidal evaluation of a variety of semisolid pharmaceuticals.

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THE ROLE OF ANTAGONISMS IN THE EVALUATION OF ANTISEPTICS

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The action of a variety of antibacterial substances and processes is reversible. It is the purpose of this paper to furnish a review of selected, typical instances of this and of related phenomena and to consider their relevance to the problem of the performance of the individual antibacterial agents. It is obvious that the antibacterial effect, by virtue of the *modus operandi* of any particular agent, or of the surrounding conditions under which it takes place, or of its duration, may produce changes within the bacterial cell of such finality that recovery is made impossible, regardless of the application of any measures potentially antagonistic to the factor or factors involved in the primary antibacterial action. It is equally obvious that what appears as bacterial death under a particular set of experimental conditions will depend largely upon the criteria applied. Frequently, a variation in these conditions will reveal a complete revival of a presumably "dead" bacterial cell, or at least a restoration of some of its biological functions.

The kinetics of bactericidal action have been studied extensively by many investigators over a period of years. For a review of this matter, reference may be made, *e.g.*, to Rahn's exhaustive treatment.[1] Whereas in the past, however, the attention of the researcher was centered upon the phenomenon of bacterial death as a function of time and of the concentration of the antibacterial agent, today's wider knowledge of cellular chemistry and physiology suggests the study of this phenomenon in terms of the individual physical, physico-chemical, and chemical reactions between the antibacterial substances and the specific constituents of the bacterial cell which are involved significantly in its biological activity. The latter subject is hardly of any interest with regard to the practical aspects of the action of those agents (disinfectants) which operate as general protoplasmic poisons. It is of great importance, however, for a consideration of the action of chemotherapeutic and antiseptic materials which act by producing a selective effect upon one or more phases of the metabolism of the bacterial cell. Obviously, the specific metabolic phases affected will vary with the antibacterial agents employed.

The study of the action of antibacterial substances may be conducted from two different angles. It may concern itself, *e.g.*, with the determination of specific enzyme systems upon which the bacterial cell relies for its functioning, the primary purpose of such an inquiry being an extension of our knowledge of cell biology. Or, it may have an essentially similar goal, but with the aspect of an eventual utilization of the results of the inquiry for prophylactic or therapeutic purposes. Special mention of these two angles is made because it is relevant to the subject of the reversibility of antibacterial action and of the means by which this reversibility can be achieved. Thus, it is obvious that, in a paper devoted to the practical aspect of the problem, the same considerations cannot apply to the kind of antagonistic or reversing agents or processes employed solely for investigational purposes, if such agents or processes do not themselves occur under the conditions of the intended practical use, or if there is no reason to assume the probability of the existence of sufficiently analogous materials or processes under the conditions of practice.

By the way of an example, reference could be made here to the use of thioglycolate in testing mercurial antiseptics. While thioglycolate itself probably does not occur in tissue or in body fluids, its use in the testing procedure appears justified because of the occurrence, under use conditions, of other thiol structures whose reactivity with mercurials bears an adequate resemblance to that of thioglycolate. By the same token, it might not be proper to require the employment, under the same conditions, of certain dithiol compounds possessed of a much greater reactivity with respect to heavy metal salts or metal-organic compounds. Their consideration would be relevant, *e.g.*, to the problem of detoxification of certain systemic arsenical poisons (such as lewisite) but hardly to that of the antagonism to mercurial antiseptics of the thiol systems occurring in the animal body.

The possibility of reversal of antibacterial action by means of reactions or of processes likely to occur under use conditions deserves considerable attention. It is essential to ascertain the condition of the bacteria, following their exposure to the action of a given antibacterial agent, in order to learn whether or not the organisms, which, in the course of a given testing procedure, seemingly have been "killed" or otherwise rendered harmless, have actually ceased to be a source of infection when placed under the more favorable conditions to be found in an open wound, on a mucous membrane, in the intestinal tract, *etc.*

In this connection, a distinction must be drawn between the portion of the antibacterial agent which may have been "transferred" with the inoculum to the subculture medium, in a quantity sufficient to produce a bacteriostatic concentration in the surroundings, and that portion which has become affixed to the bacterial cells and has initiated there a process that would ultimately lead to the destruction of their infectiousness unless interrupted by some antagonistic substance or process furnished by the animal body. In the case of true reversibility, one is concerned only with the effects of the latter fraction of the antibacterial matter.

Reversibility has been observed in the following instances: (1) desorption or elution of adsorbed antiseptics; (2) neutralization of acid by alkali; (3) inactivation of formaldehyde by ammonia; (4) inactivation of antiseptic chelating agents by metal ions; (5) inactivation of mercurials and other metallic compounds by thiols and of antibacterial thio-compounds by metal salts; (6) impairment of the action of antiseptic dyes by pH variation or ionic displacement; (7) reaction between cationics and phospholipids; (8) impairment of oxidizing agents by reducing substances; (9) antagonism between sulfonamides and para-aminobenzoic acid (and related substances); (10) impairment of the action of antibacterial metabolite analogues by essential metabolites, *etc.* The paragraphs which follow will furnish some examples of these effects, with considerations of their relevance to use conditions wherever indicated.

Desorption or Elution of Adsorbed Antiseptics. The experiments by Süpfle and Müller[2] on the detoxification of anthrax spores, ostensibly killed by mercuric chloride, are relevant to this section, as well as to a subsequent one dealing with the inactivating action upon mercurials of thio-compounds. They were undertaken with the intention of either verifying or refuting Geppert's[3] contention that reversal by ammonium disulfide of the toxic action of mercuric chloride must take place within the bacterial cell (as a result of the precipitation of the metal as sulfide) Süpfle and Müller considered it unlikely that such reversal would still be possible following a chemical reaction between the mercuric salt and some portion of the cell contents. On the other hand, they reasoned that, if the toxic mercuric chloride was merely adsorbed upon the cell wall, *i.e.*, without having participated in a chemical reaction, it should be possible to produce reversal by physical means, *e.g.*, by another adsorption process rather than chemically —by precipitating the insoluble metal sulfide.

Using activated carbon as a competitive adsorbent, they found living anthrax spores following exposure to: 5 per cent $HgCl_2$ for 11 days; 3 per cent $HgCl_2$ for 38 days; 2 per cent $HgCl_2$ for 40 days; and 1 per cent $HgCl_2$ for 40 days. When working with *Micrococcus pyogenes aureus*, they found viable cells following treatment with a one-tenth per cent solution of mercuric chloride for two hours.

From these findings, they concluded that mercuric chloride requires a long time before penetrating into the bacterial protoplasm with a fatal effect upon the cell, and that the first step in its antibacterial performance is prolonged adsorption on the cell wall.

When spores of *Bacillus subtilis* are exposed to the action of certain cationics for short periods, complete killing appears to result. The picture is not affected by subsequent treatment with anionic agents to effect neutralization. Because of the improbable character of this phenomenon, Kivela, Mallmann, and Churchill[4] attempted removal of any adsorbed surface active material by repeated centrifuging and washing with water or physiological saline. The result was a high percentage of recovery. A modification of this procedure yielded comparable results.

Reversal of the bacteriostatic action of the adsorbed surface active cations through desorption could be demonstrated also by a restoration of the negative mobilities of the *B. subtilis* spores in an electrophoretic field. "Competitive" adsorption plays a role in the reversing effect upon surface active cationic agents of agar, according to Quisno, Gibby, and Foter,[5] who obtained relatively higher plate counts from 0.1 cc. than from 1 cc. portions of the same cationic-bacteria mixture. Flett, Haring, Guiteras, and Shapiro[6] claimed that desorption of phenol from suspensions of *M. pyogenes aureus* could be accomplished by activated charcoal, causing a shift in the minimum concentration germicidal in 10 but not in 5 minutes, from 1:65 (in regular F.D.A. medium) to 1:55. Tilley,[7] however, who attempted to duplicate the work of Flett and associates, could not confirm their findings.

As to any relevance to practical antisepsis of the several findings discussed, it might well be questioned whether or not prolonged contact with tissue fluids, particularly of larger volumes, would not bring about reactivation of pathogenic spores inhibited temporarily by some adsorbed surface active agent. In the case of nonspore forming bacteria, such a contingency would be less likely, however, in view of their lower resistance, provided that an adequate concentration of the antibacterial substance was permitted to act for a sufficient period of time.

The Effect of Neutralization of Acid by Alkali. In this case, too, bacterial spores lend themselves logically for the demonstration of the above effect because of the comparatively longer time required in their case, than in that of vegetative bacteria, to bring about cessation of viability. TABLE 1 contrasts the time periods required to produce an apparent sporicidal effect on anthrax by different concentrations of hydrochloric acid (plus 10 per cent of sodium chloride) without, and with, subsequent washing, followed by treatment with a solution of sodium hydroxide. The data given in the second column were obtained by Gegenbauer and Reichel[8] and those in the third column by Müller.[9] Incidentally, in some cases, 4 per cent hydrochloric acid was not effective even in two weeks. Prior to this, however, Schattenfroh[10] claimed that a solution of 1 per cent hydrochloric acid plus 10 per cent sodium chloride represented a satisfactory disinfectant for anthrax infected skins.

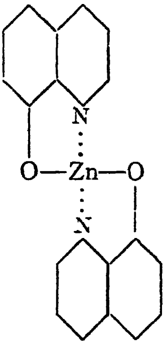
TABLE 1
SPORICIDAL ACTION OF HYDROCHLORIC ACID

% Concentration	*Req. time of action without washing and neutralization (hours)*	*Req. time of action with washing and neutralization (days)*
0.5	132	>31
1.0	64	31
2.0	16	11
4.0	5⅓	10

Certain aspects of reversal of the antibacterial action of acid and basic dyes by a pH change toward the alkaline or acid sides, respectively, belong in this section. However, the subject of dyes will be discussed under a separate heading.

Inactivation of Formaldehyde by Ammonia. Paul and Prall[11] claimed that anthrax spores are killed by a 3 per cent formaldehyde solution in 3 hours at the latest, while Xylander[12] maintained that this effect is not produced in less than 15 hours. Using ammonia as the detoxifying agent (following removal of formaldehyde by washing), Müller[9] found that 1 and 3 per cent formaldehyde solutions required a reaction period of 6 days to produce a sporicidal result.

Inactivation of Antiseptic Chelating Agents by Metal Ions. The rather unique position of 8-hydroxyquinoline and of its salts as antiseptic and antifungal agents stimulated two independent inquiries into the reasons for this behavior. First, Zentmeyer[13] suggested that the antifungal action of this class of compounds was due to the formation of chelates with trace metals essential to the functioning of certain vital cellular enzymes. He supported this hypothesis by demonstrating reversal of the antifungal effect by zinc ions. The structural formula of the zinc chelate is shown below:



Subsequently, Albert, Rubbo, Goldacre, and Balfour[14] attacked this problem, following Albert's[15] announcement of a hypothesis corresponding to that of Zentmeyer's. Of seven possible isomers, 8-hydroxyquinoline was found to be the only one displaying antibacterial properties, an outstanding example of biological specificity. This is attributed to its capacity of formation of feebly dissociated chelate complexes in which the metal becomes a part of a heterocyclic ring system. While structurally related substances with a capacity for chelation also display antibacterial activity, it is by no means true that all chelating agents are *eo ipso* possessed of antibacterial properties. Indeed, the molecular structure is no less important in this regard than the capacity for chelation. On the other hand, "cupferron" (ammonium nitrosophenyl-hydroxylamine), which forms chelate inner complex compounds, also shows fungistatic action, according to Zentmeyer.[13]

Of particular importance is the chelate strength at the physiological pH 7.3. At substantially lower pH, chelation is suppressed when the ionization of the hydroxyl group is prevented, or if the nitrogen atom is made quaternary. Both these effects also suppress antibacterial activity due to the combination of the chelating agent with an ionized metal forming a part of an essential cellular enzyme on the bacterial surface.

Reversal of the antibacterial action of chelating antiseptics may be accomplished by the addition of metal salts. In the case of 8-hydroxyquinoline, this reveals a sharp differentiation between Gram-positive and Gram-negative microorganisms. Thus, the addition of a cobalt salt alone produces growth of the former, in the presence of inhibitory concentrations of 8-hydroxyquinoline, while only zinc and iron salts reverse the inhibitory action upon the Gram-negative species.

It should be mentioned in passing that 5-methyl-8-hydroxyquinoline offers some hope for clinical usefulness, owing to its low toxicity.

Inactivation of Mercurials by Thiols and of Antibacterial Thio-Compounds by Metal Salts. The fundamental phenomenon of reversal of the antibacterial action of mercuric chloride has been known for many years. In 1881, Koch[16] praised this chemical as an almost perfect germicide. Soon thereafter, however, in 1889, Geppert drew attention to its high bacteriostatic quality, which masked its comparatively low bactericidal power.[3] He did this by demonstrating that anthrax spores retained their infectiousness for animals following exposure to mercuric chloride even though they did not germinate when subcultured. Geppert proved also that, by employing ammonium disulfide, the interfering, inhibitory effect of the mercury salt could be eliminated, with the result that a 0.1 per cent solution of mercuric chloride was shown to be incapable of killing anthrax spores even in twenty-four hours.

Similar experiments were carried out by later investigators, both with spore-forming and with vegetative microorganisms. Thus, Müller[9] found that if the anthrax spore suspension is washed prior to treatment with ammonium disulfide, the spores were not killed by 0.1 to 3 per cent solutions of mercuric chloride, even in fifty-five days. Engelhardt[17] showed that staphylococci kept in a 1 per cent solution of mercuric chloride for seventy-two hours grew after precipitation of the mercury ion as the sulfide. McCalla[18] "revived" mercuric chloride inhibited suspensions of *Escherichia coli* by bubbling hydrogen sulfide through them. Rodenwald[19] found that the microorganisms of fowl cholera kept for several minutes in a 0.1 per cent solution of mercuric chloride and washed subsequently produced fatal results upon inoculation in animals, although the attempt to grow them *in vitro* as a control failed, thereby producing the appearance of successful disinfection.

Inactivation of the *M. pyogenes aureus* bacteriophage by mercuric chloride can be suspended completely with the aid of hydrogen sulfide, according to Krueger and Baldwin.[20] Reactivation of the influenza virus B (Lee strain), inhibited originally by mercuric chloride (and by other mercurials to be discussed subsequently), was reported by Klein, Brewer, Perez and Day.[21]

It is becoming increasingly apparent that organic mercurials follow largely the same general pattern of behavior in the reversibility of their respective antibacterial activities. This has been brought out in several recent publications. Among the more significant, from the point of view of the practical use of mercurial antiseptics, are the papers by Morton, North, and Engley[22] and by Banti.[23] Although it had been recognized earlier by Shippen[24] that dilution of mercurials in the subculture beyond their bacteriostatic range would help to place their germicidal potency in a more correct perspective, Heinemann[25] proved that mere dilution of the inoculum, as required by Shippen's procedure, would not prevent falsely high germicidal figures from being obtained.

To effect a "chemical neutralization" of the mercurials tested, Morton and associates employed the thioglycolate medium suggested by Brewer[26] for the cultivation of anaerobic microorganisms and found subsequently by Marshall, Gunnison, and Luxen[27] to be capable of suppressing bacteriostasis by mercurial compounds. (This medium, slightly modified, has been declared official by the National Institute of Health[25] for sterility testing of biological materials.) The following organic mercurials were used in the test: merbromin N.F. (disodium salt of 2,7-dibromo-4-hydroxymercurifluorescein), sodium ethylmercurithiosalicylate, and nitromersol (the anhydride of 4-nitro-3-hydroxymercuri-orthocresol). All were employed in the forms of their aqueous solutions as commonly available on the market, under their respective trade names. A virulent hemolytic streptococcus served as the test organism and a ten minute period of exposure was allowed for the action of the three mercurials.

It was found that, if subculturing the mercurial-culture mixture into thioglycolate medium resulted in growth of the culture, the organisms were still capable of producing a fatal septicemia upon intraperitoneal injection into mice. Hemolytic streptococci could be isolated from the hearts' blood of the dead animals. From this, the conclusion was drawn that pathogenic microorganisms in a state of bacteriostasis continue to be infectious for the animal body, and that true bactericidal rather than bacteriostatic action is necessary to prevent sepsis.

The findings of Morton and collaborators are not altogether unexpected, in view of certain results published by other investigators. Thus, with regard to sodium ethylmercurithiosalicylate, Morgan, Simmons, and Biggs[29] observed that at 5°C. hemolytic streptococci withstood exposure to a 0.1 per cent solution of it for one week. Subculturing to thioglycolate medium yielded evidence of their viability. With regard to similar conditions encountered in the case of the other organic mercurials, reference should be made to the papers of Nye[30] and of Hoyt, Fisk, and Burde.[31] Nungester and Kempf,[32] using an "infection-prevention" method, reported that pneumococci and streptococci remained virulent following exposure for two minutes to aqueous 0.1 and 0.2 per cent solutions, respectively, of sodium ethylmercurithiosalicylate, while Sarber[33] observed a 0.1 per cent tincture of the same mercurial to be only partially effective in preventing sepsis by hemolytic streptococci (as compared, *e.g.*, with the highly effective iodine tincture). Incidentally, Nungester, Hood, and Warren[34] found that an *in vitro* procedure employing the thioglycolate medium yielded results agreeing with those of their "infection-prevention" method when applied to mercurial antiseptics.

For the sake of completeness, attention is hereby called to the papers by Brewer,[35] Powell,[36] and Cromwell,[37] contributed in response to the criticisms directed against mercurial antiseptics by Morton and collaborators. These papers do not appear to invalidate the idea regarding the essential weakness of organic mercurials *per se* as germicides and the reversibility of their antibacterial action within the animal organism. They merely tend to emphasize the adjuvant effect of the other ingredients present in the formulations of the particular commercial preparations, which impart to them qualities capable of overcoming to some extent the inherent deficiencies of the mercurials proper.

The paper by Banti[23] is deemed to be important because it supplies an *in vitro* demonstration of the ability of blood to reverse the action upon *M. pyogenes aureus* of organic mercurials (merbromin and sodium ethylmercurithiosalicylate) and of mercuric chloride, even after a prolonged contact. There is a close analogy between the pertinent antagonistic effects of blood and of thioglycolate, respectively, furnishing a cogent rationale for the use of thioglycolate in an experimental screening of antiseptics which, in practical use, may come in contact with body fluids, such as blood, serum, pus, *etc.* Certain cationic agents, as well as inorganic and organic chlorine antiseptics, appear to be subject to the antagonistic action of blood, although the effect is far less striking in their case than in that of the mercury compounds studied. No such variations were found in the case of phenol.

The "semi-micro" technique developed some time ago for the bacteriological evaluation of cationic disinfectants[71] was adapted by Klarmann, Wright, and Shternov to a study of several antiseptics, including those of the mercurial variety. Briefly, this method calls for contacting the bacterial culture with the diluted germicide for a definite period of time, then diluting the *entire* mixture with a liquid nutrient medium to which a suitable "neutralizer" had been added. (As a rule, 0.05 cc. of broth culture, 0.5 cc. of diluted germicide, and 20 cc. of medium plus neutralizer are employed.) Since the contact between the test organism and the antibacterial agent under investigation takes place in the absence of any antagonistic matter, the method admittedly tends to operate in favor of the antibacterial substance. In its present adaptation, the procedure calls for the addition of 10 per cent defibrinated horse blood to the nutrient broth used to dilute the bacteria-germicide mixture at the end of the reaction period. This modification is expected to indicate the effect of blood upon the test-organisms previously exposed to the action of the antibacterial agent, and to this extent supply some information as to the situation likely to arise, *e.g.*, in an infected wound.

TABLE 2 shows that the antibacterial action of the four mercurials tested is evidently reversed by blood, and that, as in Banti's experiments, here, too, there is a parallelism between the reversing effects of blood and thioglycolate respectively. It is noteworthy that 10 per cent serum is ineffective as a "neutralizing" agent, in that it fails to suspend the bacteriostatic action of the mercurials.

A matter of related practical relevance is that of treating surgical catgut with mercury compounds. Davis, Block, and Stonehill[38] investigated the performance of nonboilable tubing fluid, which contains phenylmercuric benzoate (0.025 per cent), in alcoholic (ethanol plus isopropanol) solution. As test organisms, they employed *Clostridium sporogenes* (representative of anaerobic spore formers), *B. subtilis* (representative of aerobic spore formers), *E. coli* (representative of aerobic Gram-negatives), and *M. pyogenes aureus* (representative of aerobic Gram-positives). Specimens of catgut infected with one of these microorganisms were transferred to different liquid media, of which the thioglycolate medium was found to be the most effective in reversing the action of phenylmercuric benzoate and in permitting growth of all test organisms within four days. Failure to "neutralize" properly the effect of the mercurial prior to incubation was responsible for the prevention of growth in eleven out of twenty-four tubes, attesting to the powerful bacteriostatic action of the mercurial and producing a deceptive appearance of sterility.

Studies on the Mechanism of Inactivation of Bacteria by Mercurials. As indicated before, the phenomenon of inactivation of bacteria by mercury compounds and their reactivation by thiol compounds has been familiar for a number of years. Its elucidation is due to several investigators, beginning with Rapkine's[39] and Fildes's[40] more recent papers, in which the antibacterial action of mercury has been attributed to a blocking of cellular thiol receptors with formation of mercaptide bonds. Its reversal is due to the cleavage of these bonds by added thiol compounds.

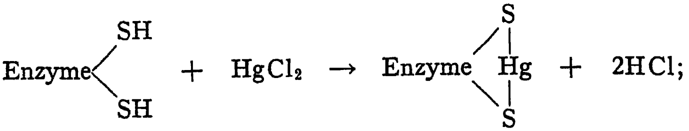
An attempt at a correlation of available thiol receptors with the required quantity of mercury to effect inhibition was made by de Loureiro and Lito,[41] who employed the thiol titration method of Kuhn, Birkofer, and Quackenbush[42] in order to determine the number of SH-groups in a bacterial suspension. They found that such inhibition is indeed a reflexion of a stoichiometrical relationship between the mercurial antiseptic and the thiol groups of the bacterial cell. This was verified by determining the survival periods of suspensions of *Salmonella typhosa* mixed with multiples and submultiples of the quantity of the mercurial antiseptics studied (mercuric chloride, mercuric cyanide, and mercuric oxycyanide, also sodium ethylmercurithiosalicylate), theoretically equivalent to the thiol content of the bacterial suspension. In the case of *S. typhosa*, it was sufficient to block only one-fourth of the titratable thiol groups in order to effect inhibition.

TABLE 2
PERFORMANCE OF MERCURIAL ANTISEPTICS BY SEMIMICRO METHOD
M. pyogenes aureus, 20°C.

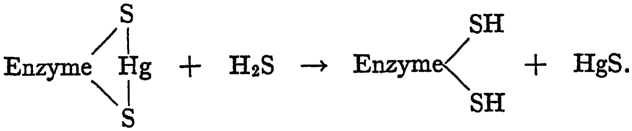
Antiseptic	*F.D.A. broth*	*F.D.A. broth + 10% blood*	*F.D.A. broth + 10% serum*	*F.D.A. thioglycolate broth*
Merbromin 1:50	–*	+	–*	+
Nitromersol 1:2500	–*	+	–*	+
Sodium ethylmercurithiosalicylate 1:1000	–*	+	–*	+
Mercuric chloride 1:1000	–*	+	–*	+

* Bacteriostasis.

The essential character of the thiol groups depends upon the fact that a large number of enzymes are "sulfhydryl" or "SH-enzymes," as shown by Barron and coworkers.[43] Compounds of mercury, as well as those of other heavy metals, are toxic because of their capacity to inhibit the functioning of these "SH-enzymes." The mercaptides formed are readily dissociable, as a rule. Almost complete reversal of inhibition could be accomplished in the case of succinoxidase so long as complete inhibition has not taken place; when the latter stage was being approached, reversal became increasingly difficult.[44] The reactions involved, respectively, in the inhibition of "SH-enzymes" by mercury and in the reversal by thiols may be illustrated by the following equations:

I)


$$\text{Enzyme}{<}\begin{matrix}SH\\SH\end{matrix} + HgCl_2 \rightarrow \text{Enzyme}{<}\begin{matrix}S\\S\end{matrix}{>}Hg + 2HCl;$$

II)


$$\text{Enzyme}{<}\begin{matrix}S\\S\end{matrix}{>}Hg + H_2S \rightarrow \text{Enzyme}{<}\begin{matrix}SH\\SH\end{matrix} + HgS.$$

Essential SH-groups occur in different enzyme proteins, such as in urease, in triosedehydrogenase, in the cathepsin group of the proteases, *etc.* Their presence may be detected by means of *p*-chloromercuric benzoate or of trivalent arsenoxide compounds, acting as mercaptide forming reagents. Their action is reversed by a number of low-molecular thiol compounds such as cysteine, glutathione, thioglycolate, *etc.* Thus, according to Bersin,[45] the inhibition of the proteolytic action of papain is accomplished by trivalent organic arsenicals, and its reactivation by glutathione.*

The reason why some "SH-enzymes" are affected by certain reagents, while others are not, probably lies in the variation of accessibility of the essential SH-groups which is characteristic for the different enzymes. The fact that a given antiseptic is known to inactivate a given enzyme, however, must not be taken to signify that this particular reaction is involved in the bactericidal or bacteriostatic effect observed. That more than one enzyme is affected by mercury appears from the work of Selzer and Baumberger,[47] who found that the oxygen consumption of yeast is completely inhibited by mercuric chloride when glucose is the substrate, but that it is unaffected when lactic or pyruvic acids serve in this function.

Generally speaking, the degree of completeness of the reversal of enzyme inhibition depends upon the dissociation constant of the inhibitor-antidote complex. If this constant is very low, as compared with that of the enzyme-inhibitor complex, a complete reactivation of the enzyme might be expected. The reversal by thio-compounds of the inhibitory action of heavy metals supplies an example of the above generalization.

Arsenicals must be converted through the animal body's reductive or oxidative processes into arsenoxides, before they can exhibit their anti-

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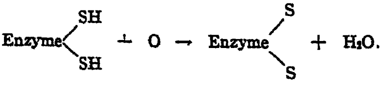
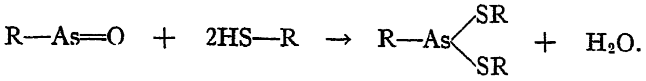
* For the sake of completeness, it may be mentioned that thiol compounds are capable also of reversing the action of certain oxidizing enzyme inhibitors, such as oxygen in the presence of metallic catalysts, iodine, potassium ferrocyanide, sodium ortho-iodosobenzoate, *etc.* which operate according to the equation:



$$\text{Enzyme}{<}\begin{matrix}SH\\SH\end{matrix} + O \rightarrow \text{Enzyme}{<}\begin{matrix}S\\S\end{matrix} + H_2O.$$

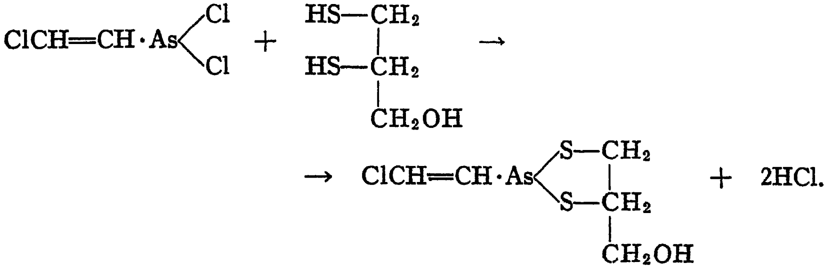
In this case, the thiols produce a reductive effect, as shown by Hellerman, Perkins, and Clark[46] in the case of crystalline urease.

bacterial action by reacting with the essential SH-groups of the bacterial cell, according to the equation:



$$R{-}As{=}O + 2HS{-}R \rightarrow R{-}As\begin{array}{l}\diagup SR\\ \diagdown SR\end{array} + H_2O.$$

Such combinations are antagonized by mono-thiol derivatives, *e.g.*, thioglycolate, thiolactate, cysteine, glutathione, *etc.* Where a stable enzyme-inhibitor complex is formed, however, as, *e.g.*, in the case of the inhibition of pyruvate-oxidase or of hexokinase by the arsenical, "lewisite," reversal cannot be accomplished by mono-thiols. In this case, a highly reactive dithiol, such as 2,3-dimercaptopropanol (BAL), is found to effect reactivation of the "SH-enzymes" mentioned, owing to its capacity to form a non-dissociating cyclic compound, according to the following equation:



$$ClCH{=}CH\cdot As\begin{array}{l}\diagup Cl\\ \diagdown Cl\end{array} + \begin{array}{l}HS{-}CH_2\\ \qquad |\\ HS{-}CH_2\\ \qquad |\\ \qquad CH_2OH\end{array} \rightarrow$$

$$\rightarrow ClCH{=}CH\cdot As\begin{array}{l}\diagup S{-}CH_2\\ \qquad\quad |\\ \diagdown S{-}CH_2\\ \qquad\quad |\\ \qquad\quad CH_2OH\end{array} + 2HCl.$$

The study of this mechanism was carried out by Whittaker upon "SH-enzymes."[48] It goes back to the fundamental work of Peters, Stocken, and Thompson.[49] It is mentioned here primarily for illustrative purposes, since it is hardly relevant to any of the processes involved in antiseptic or chemotherapeutic effects, as pointed out in the introductory remarks to this paper. It may be added parenthetically, however, that 2,3-dimercaptopropanol itself can act as an inhibitor (rather than as an antidote) for those enzymes which contain a reactive metal as a constituent part of the prosthetic group of such enzymes.[50]

In addition to its affinity for the SH-radical, the mercuric ion can enter into a combination with other reactive groupings whose presence in the living cell may be taken for granted, *e.g.*, the NH_2-group, as emphasized by Salle and Ginoza.[51]

Impairment of the Action of Antiseptic Dyes by pH-Variations or Ionic Displacement. It is noteworthy that the term "bacteriostasis" was introduced originally by Churchman in 1912 to describe the effect produced by dyes.[52] He realized that a dye may stop motility, check sporulation, or prevent cell divisions, thereby producing a state of "suspended animation."[53] The latter condition is illustrated by the phenomenon of stained anthrax spores requiring a considerably longer time than unstained spores to produce death upon injection in animals.[54]

While the conditions effecting the restoration of the viability of the anthrax organisms in the animal body are probably of a complicated character, Stearn and Stearn[55] showed that a simple change of pH was sufficient to "revive" *E coli* inhibited by basic and by acid dyes, respectively.

This is not the place for an exhaustive review of the antibacterial action of dyes. A discussion of the numerous papers published on this subject alone would by far exceed the framework of this presentation. Reference should be made, however, to the observations of Hoffmann and Rahn,[56] according to whom the bacteriostatic and the bactericidal effects of gentian violet appear to be due to different reactions. Thus, crystal violet is more bactericidal to young cells than to older ones. Also, its bactericidal effectiveness is said to be practically independent of pH, while its bacteriostatic action is greatly influenced by it. Temperature variation affects the bacteriostatic action of dyes in a manner that is different from its effect on the action of disinfectants. Gentian violet is a better bacteriostatic agent at low temperatures than at higher ones. Thus, *E. coli* requires six times as much of it at 37 as at 15°C., and for *M. pyogenes aureus* the corresponding ratio is seven to one. By contrast, in the case of a disinfectant action, an increase of temperature from 15 to 37°C. reduces the minimum effective concentration to at least one-fourth, or, more often, to an even lower fraction of the minimum germicidal concentration at 15°C.

Only scanty information is available concerning the character of the cellular constituents affected in the antibacterial action of dyes. According to Dubos[57] and Hoffmann and Rahn,[56] their action depends upon the maintenance of the redox potential of the bacterial environment above a point permitting metabolic activity. According to Stearn and Stearn,[55] however, the dyes react and combine with various groups in the cell. Thus, the more highly reactive basic dyes are assumed to be attached by the carboxyl groups, also the phosphoric groups, which participate in the functioning of the nuclear apparatus, as well as in that of certain enzyme systems.

Reversibility of the antibacterial action of dye cations through replacement with metal cations has been demonstrated by McCalla[58] in the case of methylene blue. (This phenomenon could be handled with equal reason in the section devoted to adsorption and desorption.)

Living bacteria stained and inhibited by certain dyes become decolorized and regain their viability when placed in contact with certain organic materials, such as serum, pus, *etc.*, according to Doladilhe and Guy.[59]

The inhibitory action of acridines is reversed by nucleic acid. Wagner-Jauregg[60] claimed that this is due to the formation of definite compounds between nucleic acid and the acridine dyes. Subsequently, McIlwain[61] demonstrated reversal of the inhibitory action upon *E. coli* and *Streptococcus hemolyticus* of acriflavine by nucleic and adenylic acids. He explained this phenomenon by postulating the creation of a nutritional deficiency, as a result of the inhibitory action of acriflavine, requiring for the re-establishment of bacterial growth a supply of nutrients not needed normally. Two types of materials are involved in making up for this abnormal deficiency. One type is supplied by nucleotides (such as sodium nucleate), the other by a concentrate of amino acids and partly by phenylalanine alone. Thus, this "chemotherapeutic" approach to the elucidation of the effect of acriflavin implies, among other things, that the dye deprives the inhibited organism of the function of certain essential enzymes or of the utilization of some important metabolites, a concept which found its most fruitful application in the explanation of the antibacterial action of the sulfa drugs and of the antibiotics.

The inhibition of respiration of yeast (in a glucose-phosphate medium) by three types of basic dyes, *viz.*, acridines (*e.g.*, trypaflavine), thiazines (*e.g.*, methylene blue), and triphenylmethane dyes (*e.g.*, crystal violet), is reversed by nucleic, adenylic, and adenosine-triphosphoric acid, according to Massart, Peeters, DeLey, and Vercanteren.[62] They assume that the basic dyes are bound by nucleoproteids of the yeast cell with formation of "electro-adsorption" complexes. Desorption (through ionic displacement) is effected by the cations H^+, Na^+, Mg^{++}, and Al^{+++}.

While the experimental findings considered thus far tend to indicate that a rather superficial connection between the dye and the bacterial cell is sufficient for inhibitory action, the observations by Gale and Mitchell[63] suggest strongly that a dye must penetrate the cell wall in order to interfere with at least some forms of cellular metabolism. This conclusion is drawn from the following facts: streptococci assimilate glutamic acid from external surroundings when a source of energy, such as glucose, is available; inside the cell, the glutamic acid undergoes a metabolic change of an as yet obscure character. The inhibitory action of certain triphenylmethane dyes, however, brings about an increase of the glutamic acid concentration within the cell, indicative of interference with an intracellular metabolic process.

Blood appears to have a reversing effect upon bacteria inhibited by rivanol, as shown by Klarmann, Wright, and Shternov.[113] With a 1:5000 dilution of this dye, there occurs an almost complete inhibition of *M. pyogenes aureus*, while, upon addition of defibrinated horse blood, a considerable number of bacterial colonies are produced. (The procedure employed was as follows: To two 50 cc. centrifuge tubes were added 0.5 cc. of a 24-hour broth culture and 5 cc. of antiseptic solution. After 10 minutes of contact at 20°C., the tubes were centrifuged for 30 minutes and the supernatant liquid drained off. To one tube was added 1.5 cc. of physiological saline, to the other, 1.5 cc. defibrinated horse blood. Both tubes were scraped with a platinum loop, then filled to 15 cc. with melted agar, inverted several times, and plated. The plates were incubated for 48 hours, whereupon the colonies were counted.

The likelihood of true reversal is suggested by the fact that neither plate gave evidence of containing a bacteriostatic concentration of the dye, as verified in the usual manner, by streaking the broth culture over the agar surface.

It should be noted that the 1:5000 dilution of rivanol is below the concentration recommended for practical application (1:1000). When endeavoring to demonstrate the phenomenon of reversibility experimentally, however, it may be necessary to use concentrations or exposure times, respectively, which are just short of being able to accomplish sterilization of the test culture, in order to extend the reaction time sufficiently, thereby counteracting the progress of the antibacterial effect to its irreversible stage.

Impairment of the Action of Cationic Detergents by Phospholipids and Other Organic Materials. At physiological pH, the cationic detergents display an antibacterial effectiveness superior to that of the anionic types, while nonionic detergents are not usually antibacterial in action. In analogy with the situation observed in the case of acidic and basic dyes,[64] the effectiveness of cationic detergents rises with increasing alkalinity, and that of the anionic detergents, with increasing acidity, as shown by Baker, Harrison, and Miller,[65] in the case of the former, and by Gershenfeld and Perlstein in that of the latter.[66] At neutral and alkaline pH, the cationic agents are active against both Gram-negative and Gram-positive bacteria, while the anionic agents show some activity only with respect to the Gram-positive varieties.[67] By contrast, at pH 4 the anionic detergents are about equally active against both Gram-positive and Gram-negative bacteria.[68]

The antibacterial action of quaternary ammonium compounds is interfered with by phospholipids (lecithin, cephalin, sphingomyelin) as shown by Baker, Harrison, and Miller,[65] by Gershenfeld and Ibsen,[69] and by Brewer.[70] According to Klarmann and Wright,[71] both serum and blood reduce their antibacterial efficiency to a considerable extent. Another strongly impairing agent is oxgall, which the latter authors employ, therefore, in a special method for determining the disinfectant action of quaternary ammonium compounds.[72] By contrast, protamines display a synergistic effect.[73]

In the case of "subgermicidal" concentrations, the action of cationic agents appears to be susceptible to the reversing action of blood.[113] This could be demonstrated by the same technic as has been described before (in the case of the reversal of the action of rivanol). The cationic agent employed was dimethylbenzyldodecylammonium chloride, the dilution 1:50,000. No such phenomenon is observed with subgermicidal dilutions of phenol (1:90 to 1:100), para-chlorophenol (1:400), or a saponated cresylic disinfectant (1:500).

That this phenomenon is not due to bacteriostasis has been verified in the usual manner. If the concentration recommended for practical antiseptic purposes had been applied, the above effect would not have been produced. To obtain it, it was necessary to reduce the reactivity of the cationic compound by dilution to the extent indicated.

As to the character of the interference of phospholipids with the antibacterial action of synthetic detergents, Miller, Lemon, and Miller[74] suggest that phospholipids prevent denaturation of proteins (as measured by the liberation of sulfhydryl radicals). It may be assumed that this is due to the formation with the detergents of stable complexes, with a low tendency to dissociation. The molecular size of the antiseptics, as well as of their inhibitors, is significant in determining this tendency, inasmuch as high molecular weight favors the formation of insoluble or feebly dissociating complexes.[75] Another explanation, as advanced by Baker, Harrison, and Miller,[65] is that phospholipids (which do not have the capacity of affecting bacterial metabolism) protect the cell by altering the character of the cell membrane so as to prevent the penetration of the antiseptic agent.

The successful attempt by Kivela, *et al.* to produce reversal of the inhibitory effect upon spores of cationic agents by mechanical means has been referred to before.[4] While the reversal of the bactericidal action of a cationic compound by means of an anionic agent (sodium lauryl sulfate) has been reported by Valko and DuBois,[76] it could not be confirmed either by Klein and Kardon[77] or by Weber and Black.[78] Thus, the direct effect upon bacteria of quaternary ammonium compounds would appear to be of an irreversible character. This would fit in with Hotchkiss's[79] observation as to the discharge of the cell fluids of vegetative bacteria into the suspending medium, probably as a result of the high osmotic pressure produced upon the bacterial cell wall by the adsorbed cationic agent.* (The reversal in the case of the spores is accounted for by the thickness and impermeability of their cell wall which, therefore, resists the osmotic action most effectively.)

While the cytolytic action of cationic agents is a function of their great surface activity, it must be borne in mind, however, that the latter quality alone does not determine their antibacterial efficacy, which, in general, depends importantly upon the structure of the molecule.

As to the relevance of the *in vitro* findings to the *in vivo* applications for antiseptic purposes, it appears that considerably higher concentrations of cationics must be employed than would be indicated by tests carried out without an inquiry into any antagonistic action of body fluids. This is illustrated by Sarber's[81] finding that a one-half per cent solution of a cationic antiseptic (cetylpyridinium chloride, applied, incidentally, in the form of a tincture) was not uniformly effective in preventing streptococcal sepsis (following intraperitoneal implantation of infected and subsequently "disinfected" skin portions). Pierce and Tilden[82] reported that only a one per cent solution of another cationic compound (dimethylbenzyldodecylammonium chloride) compared in its efficacy with two per cent iodine or two per cent phenol solutions, with respect to providing protection of mice against pneumococci.

Unlike the case of vegetative bacteria, the reversibility of action upon virus proteins by quaternary ammonium compounds is a matter of record Pfankuch and Kausche[83] found that virus proteins (*e.g.*, of tobacco mosaic) are precipitated by cationic compounds, and that the effect does not go beyond a complex salt formation. Reversal through dissociation of the complex is achieved by prolonged washing or, more rapidly, by raising pH above the isoelectric point of the protein. This procedure does not affect the physical, chemical, or biological properties of the virus protein. For obvious reasons, the dissociation of the complex occurs more readily when a high molecular ammonium salt (*e.g.*, dodecylpiperidinium hydrochloride) is used to precipitate the virus protein, than when a quaternary ammonium salt is used.†

For the sake of completeness, it should be mentioned that the antibacterial potency of quaternary ammonium (and of other cationic) agents is impaired or arrested by several anionic compounds (also by soaps of fatty acids), owing to a combining tendency of the high molecular weight cations of the former with the similarly high molecular weight anions of the latter. A number of special "neutralizing" agents also have been suggested. Among them is sodium naphuride (also known as suramin sodium, Germanin or "Baeyer 205"), which has been studied by Lawrence.[85] Another compound with similar action is "Tamol," recommended by Goetchius.[86] While these and similar compounds of a nonbiologic character have their place in the delineation of the antibacterial action of cationic agents, it is felt that their employment is somewhat less relevant to the problem of the antiseptic application of these agents, with the possible exception of such considerations as, *e.g.*, the antagonism between soaps and cationic germicides in connection with the "degermation" of the skin in surgical procedures.

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* According to Gale and Taylor,[80] phenol, too, acts like a surface active agent in causing cytolysis, measurable by the release into the surrounding medium of individual aminoacids (glutamine, arginine, lysine).

† An interesting case of reversal of antibacterial action by phospholipids has been observed in the case of an inhibitory basic
lipoprotein which dissociates the basic protein away
restores its viability.[84]

For the same reason, a limited relevance to the broad problem considered here may be claimed for the otherwise highly significant findings of McCulloch and coworkers[87, 88] as to the antagonistic action upon cationic compounds of milk and of other externally occurring forms of organic matter.

Impairment of Oxidizing Agents by Reducing Substances. A direct demonstration of the antagonistic effects of chlorine and sodium thiosulfate was supplied by Müller.[9] Anthrax spores exposed to the action of chlorine, but treated subsequently with sodium thiosulfate, appear to survive when kept in a one-and-one-half per cent solution of the former for five hours; but they are killed in eight hours. A one-half per cent solution is sporicidal in twenty-one hours, but not in eight hours.

These results should be contrasted with those of Krönig and Paul,[89] who claimed that a 0.03 per cent chlorine solution is sporicidal in two minutes, and with those of Geppert,[3] who considered a 0.2 per cent solution sporicidal in fifteen seconds. Hilgermann and Marmann[90] stated that a 0.27 per cent chlorine solution was effective in less than one hour, although a 0.13 per cent solution was not effective in the same period of time.

The experiments of Mudge and Smith[91] indicate the existence of a moderate bacteriostatic action upon milk-borne microorganisms of chlorine which can be counteracted by sodium thiosulfate, as well as by a dilution factor.

As in the case of an antiseptic dye and in that of a cationic agent, so also in the case of a "sub-germicidal" concentration of a hypochlorite (1:150 dilution of a preparation containing 0.9 per cent of active chlorine), reversal of the antibacterial effect may be demonstrated using the procedure outlined by Klarmann, Wright, and Shternov.[113]

The same considerations as to its practical significance apply in this instance as in the two instances dealt with previously.

Of considerable interest to the problem under discussion, is Flemming's[92] observation on the antibacterial performance of the Carrell-Dakin solution which, some time ago, enjoyed considerable acceptance. When introduced into a wound, its antibacterial potency (as determined by its available chlorine content) drops to a point below any inhibitory level within ten minutes or less. Since the pertinent technique calls for the instillation of this preparation every two hours, it follows that, for the better part of this period, the wound is without antiseptic protection. The well-demonstrated effectiveness of the Carrell-Dakin technique indicates, therefore, that the value of a preparation applied for reasons of its presumed antibacterial quality may, unexpectedly, reside in some other property. In this case, it is probably the intensified transudation, causing a good drainage and an accelerated replacement by fresh lymph, which is involved in the positive results attendant upon the application of the Carrell-Dakin method of wound therapy.

Antagonism Between Sulfonamides and Para-Aminobenzoic Acid (and Related Substances). The exhaustive treatment which this subject has received in recent years applied more to the field of chemotherapy than to that of antisepsis. It is felt, however, that some reference to it merits consideration in a paper dealing with the impairment or reversal of antibacterial action by antagonistic substances of physiologic origin, particularly since the sulfonamides have been considered variously for use as antiseptic agents.

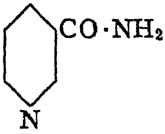
The inactivation by peptone of the bacteriostatic action of sulfanilamide was observed by Lockwood.[93] Shortly thereafter, Stamp[94] reported that an extract from a culture of hemolytic streptococci was considerably superior to peptone in bringing about such inactivation. He suggested that this extract contained a substance which was either an essential bacterial metabolite or an essential component of a bacterial enzyme. About the same time, Fildes[95] advanced the hypothesis that the metabolite in question was para-aminobenzoic acid (PABA). These developments preceded the brilliant work of Woods,[96] who identified PABA as the antagonist of sulfanilamide, and who also found that, out of some sixteen organic compounds tested, only two, *viz.*, para-hydroxyaminobenzoic acid and novocain, produced an effect analogous to that of PABA. About the same time, Selbie[97] reported that mice could not be protected against a streptococcal septicemia by sulfanilamide if PABA was administered simultaneously.

It is now known from the work of Woods, and from that of Fildes[98] and of McIlwain,[99] that bacterial growth inhibited by certain benzenoid sulfonic acids, or their amides or substituted amides, can be restored by the further addition of certain corresponding carboxylic acids or their derivatives. In the specific case of the antagonism between sulfanilamide and PABA, a widely held hypothesis as to the underlying reaction mechanism attributes this phenomenon to a competition of the drug (*i.e.*, sulfanilamide) with PABA at some essential stage of the bacterial metabolism in which PABA is involved This may be generalized to include all competitive inhibition which occurs when a given enzyme system reacts with any "foreign" compound that is structurally related to the natural substrate. If the former's structure is such that it can be reacted by the enzyme like its natural counterpart, but at a slower rate, then the cell may utilize it in its metabolism as an inferior substitute. This may express itself visibly, *e.g.*, in a reduced rate of proliferation. If, however, the "foreign" analogue is of such structure that its enzymatic utilization is impossible, it will inhibit the function of the enzyme and may ultimately stop bacterial multiplication altogether.

Antagonism Between Metabolites and Their Analogues. A number of instances have been reported which are illustrative of the broader aspects of the last statement. McIlwain,[100] who was interested in the series of the alpha-aminosulfonic acid analogues of the amino-acids: glycine, alanine, valine, leucine, beta-alanine, and aspartic acid, found that the alpha-aminosulfonic acids delay or prevent bacterial growth in chemically defined media, and that their inhibitory action can be reduced or suspended by adding alpha-aminocarboxylic acids which, incidentally, need not correspond to their inhibitory sulfonic analogues. Thus, the inhibition of *Proteus vulgaris* by alpha-aminoisobutanesulfonic acid is partly reversed by glycine or alanine, although the most complete reversal is produced by the corresponding amino acid, valine. It is noteworthy that, if *M. pyogenes aureus* is made independent, by training, of the added aminocarboxylic acids, it also loses its susceptibility to inhibition by the alpha-aminosulfonic acids.

While indole-acrylic acid acts as an inhibitor for *S. typhosa*, the addition of a trace of tryptophan restores growth. From this, Fildes[98] concludes that the action of the inhibitor is more likely to be concerned with the inhibition of the synthesis of tryptophan than with its utilization. This conclusion bears upon Woods's[96] opinion that, in the case of PABA, it is its utilization rather than its synthesis which is counteracted by sulfanilamide.

Another reaction paralleling the antagonism between PABA and the sulfonamides is that between nicotinamide (I) and pyridine-3-sulfonamide (II) as studied by McIlwain.[100] The structural similarity supplying the reason for the antagonistic behavior of the two compounds appears from their formulas below:



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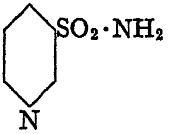
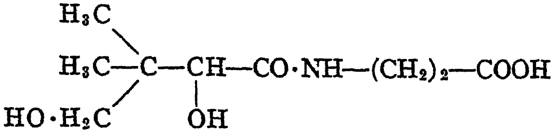


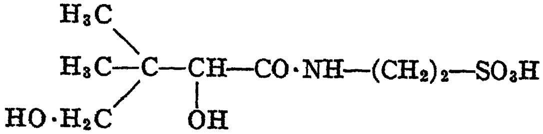
II

Snell[101] explored the reversal by pantothenic acid (III) of the inhibitory action of pantoyl taurine (N-α,γ-dihydroxy-β,β'-dimethylbutyryl taurin) (IV). Here the structural relationship is illustrated by the formulas:



$$\mathrm{H_3C{-}\underset{\underset{HO\cdot H_2C}{/}}{\overset{\overset{H_3C}{\backslash}}{C}}{-}\underset{\underset{OH}{|}}{CH}{-}CO\cdot NH{-}(CH_2)_2{-}COOH}$$

III

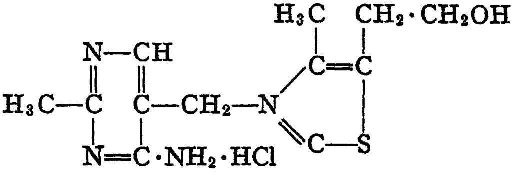


$$\mathrm{H_3C{-}\underset{\underset{HO\cdot H_2C}{/}}{\overset{\overset{H_3C}{\backslash}}{C}}{-}\underset{\underset{OH}{|}}{CH}{-}CO\cdot NH{-}(CH_2)_2{-}SO_3H}$$

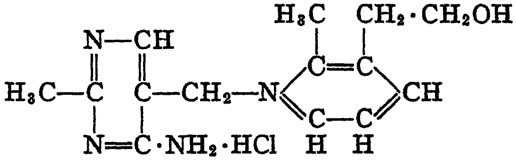
IV

Microorganisms which produce pantothenic acid in the course of their biological activity are not affected by pantoyl taurine, as shown by McIlwain.[102]

While the examples given thus far refer to the antagonistic behavior of metabolite analogues carrying a sulfonic acid group in the position in which the metabolite itself carries a carboxyl group, the experiments of Woolley and White[103] and of Wyss[104] deal with the effect of the replacement in the thiamine (V) molecule of the "natural" thiazol ring by the "unnatural" pyridin ring (yielding pyrithiamine VI). The structural formulas in question are:



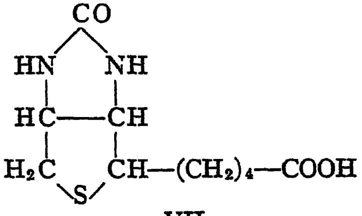
V



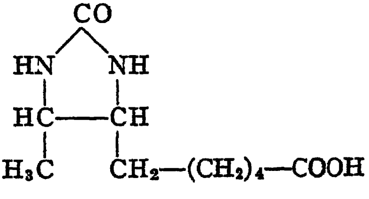
VI

It was found that pyrithiamine inhibits the growth of those bacteria which require thiamin as an essential nutrient, and that reversal of this inhibition is accomplished by means of a sufficiency of thiamin.

A somewhat related phenomenon is that of the antagonism between biotin (VII) and desthiobiotin (VIII), whose formulas are given below:



VII



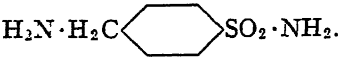
VIII

The antibacterial action of desthiobiotin upon *L. casei* was studied by Dittmer, Melville, and DuVigneaud.[105]

In all these cases, the effect of the inhibitory metabolite analogues appears to establish a state of nutritional deficiency which can be suspended or reversed by supplying to the culture an excess of the corresponding metabolite. This hypothesis, as advanced by McIlwain,[106] is in contrast with other hypotheses mentioned before, according to which the phenomenon in question depends upon the prevention of the synthesis of an essential metabolite or upon competition with a metabolite for an enzyme system vital to the proper functioning of a bacterial cell.

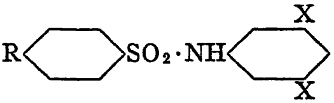
McIlwain's hypothesis has been supplemented more recently by Lampen and Jones[107] with respect to the action of sulfonamides. These authors claim that the sulfonamides interfere with the bacterial production of pteroyl derivatives of PABA (which are related to folic acid).

Modification of the molecular structure may produce an antibacterial compound which is sufficiently dissimilar to a metabolite to be unaffected in its presence. This is the case with "Marfanil" (also known as "Sulfamylon"), which resembles sulfanilamide, except that its primary amino group is not of an aromatic character, as shown by the formula below:

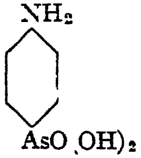


This compound, unlike sulfanilamide (and other sulfonamides), is not interfered with in its action by PABA, as proved by Schreus[108] and confirmed subsequently by McKee, Hamre, and Rake[109] and by Lawrence.[110]

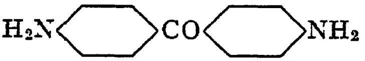
Halogenated sulfonamides of the type



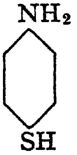
(where X is a halogen), are bacteriostatic in action, yet are not necessarily antagonized by PABA, even if R is not an aromatic amino-group, according to Goetchius and Lawrence.[111] Thus, the principle of competitive inhibition would appear to apply only to those sulfonamides which possess a free aromatic amino group. It applies also to those aromatic amines in which the sulfonic radical is replaced by other acidic radicals as, *e.g.*, in atoxyl (I), 4,4′-diaminobenzophenone (II), and aminothiophenol (III):



I

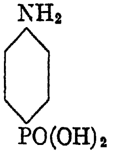


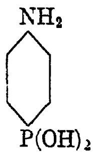
II



III

All show bacteriostatic action upon some micro-organisms which is reversed by PABA. According to Kuhn, Moeller, and Wendt,[112] the slight bacteriostatic action of phosphanilic acid (IV) is reversed by PABA. With this as a premise, and proceeding on the assumption that the strength of the sulfonamide-enzyme complex is increased with decreasing acidity of the acid radical, Klotz and Morrison[113] prepared and tested para-aminobenzene phosphorous acid (V) for its antibacterial behavior. As expected, they found it only slightly less active than sulfanilamide; and PABA neutralized this action at concentrations approximately equal to those required to counteract the action of sulfanilamide.


IV


V

Supplementary Comment

In discussing a subject such as that of this presentation, one should be aware of the delineation of the terminology used. The death of a bacterial cell may not mean the same thing to different investigators, and the same is true of terms such as inhibition, bacteriostasis, *etc.* From the applied point of view, as carried through in this paper, permanent loss of reproductive capacity would be equivalent to the cell's death. If this happens in a "reasonable" period of time, one might speak of "germicidal" action. It is evident that the qualifying term "reasonable" will depend upon the conditions under which the antibacterial agent is employed. By the same token, an antibacterial action culminating in sterility within, *e.g.*, twenty-four hours, might be regarded as either inhibition or sterilization, depending upon the circumstances under which it occurs. As pointed out by Marshall and Hrenoff,[114] a rate of multiplication greater than zero but less than normal might be descriptive of inhibition, and any rate less than zero, of germicidal action. With respect to the former, it should be remembered that retardation of multiplication can take place in two ways, *viz.*, by a decrease of the rate of multiplication or by an increase in the lag period (*i.e.*, by the time period normally required by the transferred cells to adjust themselves to their new environments). Rahn[1] showed that certain dyes may increase this period to several days without reducing the reproduction rate, once multiplication has started.

With respect to "true" germicidal action, the matter of continuing enzyme activity is still controversial. In the case of heat action, enzyme activity was observed in bacterial cells which have lost the capacity of multiplication.[115] In the case of chemical action, a considerable number of papers with contradictory conclusions have been published, of which only those by Bucca[116] and Sevag, Shelbourne, and Mudd[117] are mentioned here, by way of illustrations. Roberts and Rahn[118] showed that enzyme activity, in so far as it can be isolated experimentally, is not affected in the same manner by different antibacterial agents. Thus, in the case of *E. coli* (grown on a special synthetic medium, with sodium acetate providing the only source of energy), phenol, at the retarding concentration, caused no damage to the enzymes studied, while at the inhibitory concentration it inactivated oxidase, but not dehydrogenase. In the case of mercuric chloride, enzymatic activity was not affected at complete inhibition, and the same was true of cetylpyridinium chloride. By contrast, sodium azide (which was not germicidal as a one per cent solution) inactivated catalase at a low concentration but not oxidase or dehydrogenase, even at inhibitory concentrations.

While the pragmatic approach to the complete solution of the problems of antibacterial action and its antagonists must lead *via* the study of the enzyme reactions involved, the above findings, made in the case of comparatively simple reagents and reactions, tend to indicate the complex character of the conditions to be encountered in any pertinent research.

Conclusion

It is a well-established fact that many, if not most, antibacterial agents give the appearance of effectiveness when allowed to act upon test organisms grown on artificial laboratory media (*i.e.*, without contact with animal tissue or body fluids). It is, however, equally true that the situation is different, often radically so, when such contact is present.

A consideration of the numerous instances of competition between antibacterial agents on one hand, and their natural antagonists on the other, suggests a careful inquiry into the circumstances surrounding the intended application of the former.

Although the exact mechanism of action of the different antibacterial agents has not been elucidated, adequate cytological information is available in many instances, making it possible to equip the laboratory methods of testing antiseptics (and other antibacterial agents) with those features which would make the results of such tests more relevant to the problems posed. Among other things, this would appear to call for the abandonment of the formalistic tendency which, in the past, has been characterized by the frequent representation of a single testing procedure (with or without minor modifications) as being sufficient to handle the many aspects of evaluation of the products and processes under discussion.

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ANTISEPTICS UNDER THE FOOD, DRUG, AND COSMETIC ACT

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The Food and Drug Administration frequently receives inquiries from representatives of industry and others, seeking information relative to test methods capable of establishing definitely the status under the Food, Drug, and Cosmetic Act of drugs represented as antiseptics. We should like to offer categorical answers to such inquiries by citing precise testing methods adequate for the purpose. Unfortunately, present scientific knowledge does not permit definitive answers to the many and varied questions arising. A brief review of the contents of this monograph reveals that all of us are striving to reach just such a goal.

The merits and demerits of current testing methods will not be discussed here, but, rather, the attitude of the Administration regarding the evaluation of antiseptics under the Food, Drug, and Cosmetic Act will be briefly reviewed. The concepts discussed are not new or original and were presented by the late Dr. A. C. Hunter of the Administration in his article titled "The Evaluation of Antiseptics," which appeared in the Journal of the American Medical Association for January 2, 1943.

Much confusion has arisen as a result of the statement of Dr. Hunter in that article, and of similar statements by other representatives of the Administration, that "there is no single 'standard' or 'official' test for the evaluation of antiseptics." It is unfortunate that such a statement had to be made, and must now be repeated. Certainly, no organization would profit more from the possession or development of appropriate, simple *in vitro* procedures capable of revealing the efficacy of products represented as antiseptics. Enforcement problems would be greatly simplified and facilitated. Experience has demonstrated that the problem of product evaluation is not so easily solved. *In vitro* activity is not related directly to efficacy under conditions of use.

In vitro test procedures can, and often do, provide helpful information regarding the inherent antibacterial properties of substances, but the findings cannot be translated into terms of therapeutic efficiency. Let us consider an example in the field of antibiotics. No one will dispute that recognition of the antibacterial activity of penicillin, *in vitro*, led eventually to the discovery of its therapeutic effect *in vivo*, or that *in vitro* assay procedures have served as valuable measures for controlling the potency of penicillin preparations. It is equally clear, however, that *in vitro* activity is not a suitable or accurate index of the therapeutic value of the penicillins. Thus, penicillin X, which shows the lowest *in vitro* activity, 900 units per mg., shows the greatest activity *in vivo*, whereas penicillin K, with the greatest *in vitro* activity, 2,200 units per mg., is relatively ineffective *in vivo*. It is inescapable that such disparity of *in vitro* and *in vivo* activity is not limited to the penicillins. Favorable response to an *in vitro* test is not indicative that a substance is efficacious for the purposes for which it may be recommended, and, hence, is not sufficient to demonstrate compliance with the requirements of the law.

Articles intended for use in the diagnosis, cure, mitigation, or prevention of disease in man or other animals are classed as drugs within the meaning of the Act. Section 201 (*o*) of the Act states: "The representation of a drug, in its labeling, as an antiseptic, shall be considered to be a representation that it is a germicide, except in the case of a drug purporting to be, or represented as, an antiseptic for inhibitory use as a wet dressing, ointment, dusting powder, or such other use as involves prolonged contact with the body." Other sections of the law define a drug as misbranded if its labeling is false or misleading in any particular or unless its labeling bears adequate directions for use. Section 201 (*n*) provides that "in determining whether the labeling is misleading there shall be taken into account (among other things) not only representations made or suggested . . . but also the extent to which the labeling fails to reveal facts material in the light of such representations. . . ." Such legal terminology, of course, requires administrative and judicial interpretation. Reduced to a simple statement of fact, however, the law requires that an article represented as an antiseptic be effective in the cure or prevention of disease when used as directed and for the purposes recommended in its labeling. Such a determination must be, and is, the objective of the Food and Drug Administration in the evaluation of antiseptics subject to the provisions of the statute. It should be and, we believe, often is the objective of the manufacturer.

The task of evaluation, either by the Administration or the manufacturer, is not easy and becomes a matter of research rather than of routine analysis. It will require the collection of all related facts, by the application of methods presently available and others perhaps not yet developed, to show not only the inherent antibacterial properties of a product but the degree of efficacy with which these properties are applied under the conditions of use.

The individual manufacturer, with relatively few products to investigate, is favorably situated and has the responsibility to evaluate his own products critically. The task of the Administration is more difficult, with great numbers of varied products requiring study. The reports presented in this monograph and the interest shown by many investigators in fundamental research on antibacterial agents promises real progress and hope, on our part, that our regulatory control obligations will be lightened.

PRESENT TRENDS IN METHODS OF TESTING ANTISEPTICS

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There has been a very definite trend during the past few years in favor of *in vivo* testing of antiseptics in place of the *in vitro* methods used in the past. For obvious reasons, this is a trend in the right direction, and it will be my purpose to discuss the various procedures recently proposed.

Although the *in vitro* tests which have been and still are widely employed have not been designed to simulate *in vivo* conditions, they are useful for determining germicidal activity under specified conditions. These conditions have deliberately been made severe, so that the results of such tests could be interpreted in terms of definite values. Preparations which pass these tests are considered usefully germicidal, while those not passing the tests are either of no value or of doubtful or limited usefulness.

It would, therefore, seem logical to discuss the recent trends in methods of testing antiseptics by first considering the advantages and limitations of present procedures so generally used in this country. The recently suggested *in vivo* methods can then be discussed as possible additions to or possible replacements of present *in vitro* tests. A further possibility is a group of tests or a panel of the most useful of these procedures, which could be employed for more accurately estimating the practical value of antiseptics.

For the purpose of this discussion, the following practical definition is used: antiseptics are substances which, when applied to microorganisms, will render them innocuous either by killing them or preventing their growth, according to the character of the preparation or the method of application. This term is used especially for preparations applied to living tissue.[1] In other words, an antiseptic must act "against infection." Essentially the same definition has been included in the Federal Food, Drug, and Cosmetic Act[2] as follows: "The representation of a drug, in its labeling, as an antiseptic, shall be considered to be a representation that it is a germicide, except in the case of a drug purporting to be, or represented as, an antiseptic for inhibitory use as a wet dressing, ointment, dusting powder, or such other use as involves prolonged contact with the body."

If an antiseptic acts "against infection," it can do so either by killing infectious microorganisms or by preventing their growth. The most certain means for acting "against infection" is actually to kill the bacteria present. Antiseptics which act in this manner are called germicides. If, on the other hand, a drug is of such a nature, or is used in such a way as simply to prevent the growth of bacteria, and by so doing prevent infection, it can also be classed as an antiseptic. These latter are really bacteriostatic agents and not germicides, but they justify being classed as antiseptics because, when used clinically, they act "against infection." It is, therefore, not inconsistent to give a double meaning to this word. Many English words have double meanings, and the word "antiseptic" is a good example.

The present standard methods of testing antiseptics used in this country were designed to meet the double meaning of this word. Products classed as antiseptic may be either germicidal or bacteriostatic agents. Our standard laboratory methods of testing are designed to test for both kinds of antiseptic activity. In other words, our present standard methods of testing antiseptics are based on the definitions given. They were developed by the U. S. Food and Drug Administration[1, 3] and have been used for the testing and control of this class of drugs. Although other procedures have been suggested from time to time, these F.D.A. methods are still employed.

Since 2 per cent phenol is so generally recognized as an effective antiseptic, it serves well as a standard of comparison. However, antiseptics are not compared to phenol as in the phenol coefficient of disinfectants; they are simply *germicidally equivalent* to 2 per cent phenol. The F.D.A. test classifies antiseptics as those that are at least equal to 2 per cent phenol and those that are not. In other words, products which pass this test may be considered antiseptics.

The general use of this test made it possible to standardize antiseptics on a sound basis. As a result, the control of antiseptics has been made more effective. Also, this effective control assures the professions and the public of antiseptics of known germicidal efficiency. This has repeatedly been confirmed by comparative studies by laboratory and practical tests as well as by clinical experience in actual use of antiseptics passing the F.D.A. tests.

Recently, criticism has been made of the F.D.A. method of testing liquid antiseptics because the test does not simulate practical conditions of use. The test was never intended to duplicate clinical conditions for the simple reason that no single test can possibly simulate all the clinical conditions for which antiseptics are used. Since it is impossible to simulate all conditions, the best that can be done is to use a test that has been proved by actual clinical experience.

Within the past ten years, a number of new methods for studying and testing antiseptics have been proposed. One of the most promising of the proposed methods is an *in vivo* test recommended as an "infection prevention test" for evaluating skin antiseptics by Nungester and Kempf.[4] Test organisms are applied to the tip of the tail of white mice, after which the tail is dipped into the antiseptic, and the antiseptic is allowed to act for 2 minutes. Then the tip of the tail is amputated and inserted into the peritoneal cavity of the same mouse. The results obtained are consistent and are not subject to the variables in other *in vivo* tests. Much can be said in support of this test for determining the value of antiseptics applied to the unbroken skin.

The principal objection to this test, however, is the nature of the test organisms used. For this test to be effective, organisms pathogenic for mice must be employed. One of the organisms used is the pneumococcus, because white mice are especially susceptible to intraperitoneal injection of this organism. Actually, however, the pneumococcus is not normally present on the skin, is not responsible for skin diseases, and is not a factor in postoperative infections. For this reason, results obtained with this test organism could not be interpreted in terms of practical value for human use. Also, the pneumococcus is a very weak organism and is easily killed by antiseptics. A skin antiseptic which would pass this test by killing the pneumococci would not necessarily kill the staphylococci which are normal inhabitants of the skin and the most common cause of wound infections. Finally, the test does not contain a safety factor.

According to results obtained by this test, tincture of iodine 2 per cent was the only antiseptic that proved completely effective. While tincture of iodine is recognized as an effective skin antiseptic, satisfactory clinical results may be obtained with a number of other antiseptics. Therefore. results obtained by this test are not fully supported by clinical experience.

The only other pathogenic organism used in this "infection prevention test" is a hemolytic streptococcus. This organism is an inhabitant of the skin and is sometimes a cause of postoperative infection. Hemolytic streptococci are much weaker than staphylococci, and more easily killed by antiseptics. In fact, both penumococci and hemolytic streptococci are only about half as resistant to germicides as *Micrococcus pyogenes* var. *aureus*. Here again, there is no assurance that the killing of hemolytic streptococci will mean that staphylococci will be affected. There is no margin of safety in the test, because weak test organisms are employed.

This infection-prevention method of testing skin antiseptics of Nungester and Kempf[4] is the most suitable proposed so far, in spite of these deficiencies. This method did not, however, provide for testing toxicity. Spaulding and Bondi[5] have provided such a toxicity test in connection with it, and this combined method offers so much promise as a possible standard procedure that further analysis and evaluation is justified.

In the first place, the test is simple and requires neither special techniques nor special equipment of any kind. It can therefore be performed in any bacteriological laboratory. The results obtained are definite and readily reproducible, and, with reasonable caution, can be interpreted in terms of practical values. Also, proper evaluation of skin antiseptics can only be assured by such a comparison of infection prevention and toxicity.

Using the Nungester-Kempf procedure, the highest dilution of the antiseptic preventing infection in one-half of the mice is designated as the infection-prevention index (IP/50). Using the same technique, but eliminating the use of the test organisms, the greatest concentration of the antiseptic which fails to kill one-half of the mice is designated as the toxicity index (T/50). Since the same *in vivo* technique is used in both tests. direct comparisons of the two are justified. Then IP/50 divided by T/50 can be designated as the infection prevention-toxicity index (IP-T/50), which indicates the difference between bactericidal activity and toxicity. Consequently, the higher the IP-T index value of the skin antiseptic, the more satisfactory it should be for practical use under clinical conditions.

Spaulding and Bondi used as the test organism a type I pneumococcus of such virulence that 5 to 13 were lethal for mice by intraperitoneal injection. Using this organism, tyrothricin gave an IP-T index of 12,000 and Compound G4, 30, whereas Compound G214 gave an index of 2, Zephiran, 4, iodine, 4, and Metaphen, 3. Although these compounds are used as skin antiseptics, they are also widely used for application to cuts and wounds with consequent opportunity for absorption.

For this reason, and also since there is no practical and reproducible method as yet for evaluating skin toxicity, the IP-T index may well be used for evaluating antiseptics generally. In support of this method, it must be emphasized that infection-prevention and toxicity are obtained by the same technique and that these are *in vivo* tests. It has also been shown that results obtained by this method are comparable to those obtained by the phagocyte-toxicity method of Welch and Hunter, as well as by the LD/50 intraperitoneal method. The toxicity relationships, as shown by Spaulding and Bondi, are relatively in general agreement by these methods.

The principal objection to this method is again the use of the pneumococcus as the test organism. Realizing this, Spaulding and Bondi attempted to use the Smith strain of a staphylococcus in a series of infection prevention-toxicity tests. These tests were not satisfactory, since the survival rates were inconsistent and not reproducible. They suggested the possibility of using the mouse-virulent Smith strain in such tests, provided the cultures were swabbed on the tail, mucin introduced into the abdominal cavity, and the test mice cultured for staphylococci after 2 or 3 days without waiting for death to occur. It is evident that this organism should be used if possible, since it is a common skin organism and the most common cause of infection in cuts, wounds, and skin injuries generally.

Similar attempts were made by Spaulding and Bondi to use a streptococcus, with results, in general, intermediate between those obtained with the pneumococcus and staphylococcus. Here again, the streptococcus is less resistant to germicides than the staphylococcus and should not be used in testing antiseptics, if it is possible to use a staphylococcus. In spite of these objections and the lack of certain detailed specifications, the infection prevention-toxicity method of Spaulding and Bondi appears to be the best *in vivo* method for evaluating antiseptics that has so far been suggested.

There have been many suggestions that the laboratory testing of general antiseptics simulate practical conditions of use. Such tests should, if possible, also indicate the degree of toxicity of antiseptics for human and animal tissue. Efforts have been made to devise tests combining these two features, germicidal activity and toxicity.

Among the first such methods presented was one which made use of a "toxicity index," obtained by exposing both embryonic chicken heart tissue and a culture of *M. pyogenes* var. *aureus* to an antiseptic, in order to compare inhibition of tissue growth with germicidal action.[6] While this is a very interesting method, its use is limited to research. It is an *in vitro* method that does not simulate practical conditions of use of antiseptics.

In the first place, embryonic chicken heart muscle is not comparable to human or animal tissue. No known relationship exists between this tissue and skin, mucous membranes, or areas surrounding wounds. Toxicity to embryonic chicken heart muscle does not mean equivalent toxicity to epithelial tissue, for example. Furthermore, results obtained by this "toxicity index test" do not properly classify antiseptics in terms of usefulness under clinical conditions. For example, it was necessary to use a 1–1800 dilution of iodine in order to arrive at a "toxicity index" for this antiseptic. However, in practice, 2 per cent tincture of iodine is used as an antiseptic. Actually there is no clinical relationship between the toxicity to tissue of a 1–1800 dilution of iodine and of a 2 per cent or 7 per cent tincture.

The following are valid objections to this "toxicity index test" procedure: (1) embryonic chicken heart muscle does not represent human or animal skin, mucous membrane, or tissue exposed in wounds; (2) the test requires exposure of the tissue to antiseptic for 48 hours, whereas in actual use, antiseptics are ordinarily applied for much shorter time periods—in some instances only minutes; (3) concentrations of the antiseptics used in this test are weaker than concentrations used in practice; and (4) the conditions of the test do not in any sense simulate the clinical conditions under which antiseptics are used. Therefore, the results obtained by this test cannot be interpreted in terms of practical value.

Another suggested method of evaluating antiseptics by comparing germicidal activity with toxicity is one in which leucocytes and skin are employed as tissue.[7] This method, published at about the same time as the above "toxicity index" method, has many desirable features and approximates clinical conditions more closely than many other laboratory test procedures. This method is a combination of (1) germicidal tests by the F.D.A. method for liquid antiseptics, (2) diffusibility tests through paralodion sacs, (3) toxicity test against leucocytes, and (4) tests for intradermal toxicity.

The object of this test is to determine which antiseptics are best suited for use in wounds, cuts, abrasions, and cavities—in other words, for surgical use. The ideal antiseptic for surgical use should possess positive germicidal activity against pus-forming organisms, should be active in the presence of serum and blood, should be diffusible—that is, should penetrate into body tissue—should be relatively nontoxic in the dilutions employed, and should be inexpensive. The author of this test has considered all of these factors in this study.

In this test, the standard Food and Drug Administration Method for Liquid Antiseptics is used both with and without blood serum, for determining germicidal activity. Diffusibility tests are made with paralodion sacs, the antiseptics being placed inside the sacs, and the dialysate tested for germicidal activity.

The test for toxicity is unique. White blood cells (leucocytes) constitute an important defensive factor against infection. When they are killed by a germicide, they are prevented from accomplishing their normal function of engulfing and killing infectious bacteria. This test is one which shows whether or not white blood cells are killed by the germicide used, in the dilution usually employed.

In some respects, this test is superior to the first "toxicity index test" described. In the first place, leucocytes and human skin are employed rather than embryonic chicken heart tissue. Also, exposure times to the tissue and to the test bacteria (*M. pyogenes* var. *aureus*) are comparable, not widely different, as in the first "toxicity index test." The test for penetration through blood serum is of much importance, and results obtained are of significance in terms of practical value.

In 1940, a second method for computing a "toxicity index" by the use of leucocytes was suggested.[8] According to the authors, "In the development of the proposed method an attempt has been made to approach as nearly as possible the conditions under which antiseptics are used." The method proposed resembles the one just discussed, in that leucocytes are used for testing toxicity, but it fails to meet the authors' promise to simulate conditions under which antiseptics are actually used. In fact, it does not approach this ideal as closely as the first leucocyte method discussed, since it makes no provision for testing for toxicity against such tissue as skin, nor does it test for diffusibility and penetration of tissue by antiseptics.

Under the conditions of this test, the leucocytes and the test organism, *M. pyogenes* var. *aureus*, are exposed to the antiseptic for 30 minutes. Observations are made as to the ability of the leucocytes to phagocytize the staphylococci. The "toxicity index" is determined by dividing the highest dilution "toxic" for the leucocytes by the highest dilution killing the test organism. If presence of the antiseptic inhibits phagocytosis in a dilution greater than the germicidal dilution, the "toxicity index" is above 1. If the dilution inhibiting the leucocytes is more concentrated than that killing the test organism, the "toxicity index" is less than 1. In this connection, it is of interest to note that inhibition of phagocytosis is designated as "toxicity to tissue" and not simply as inhibition of leucocytic activity. Also, the figure obtained by dividing the dilution inhibiting phagocytosis by the dilution killing *M. pyogenes* var. *aureus* is designated as the "toxicity index."

As explained earlier, although leucocytes are tissue, they do not represent those body tissues to which antiseptics are ordinarily applied. They do not possess a resistance to antiseptics and other drugs that is comparable to such resistance by epithelium, mucous membrane, *etc.*

In another paper,[9] it has been shown that inhibition of leucocytes by antiseptics is due to destruction of opsonic complement, or opsonins. Since opsonins, normally present in the blood, are necessary in order for leucocytes to kill bacteria, anything which destroys this substance will prevent phagocytosis. Opsonins are nonliving; therefore, their destruction is not due to toxic action. If chemical alteration (destruction) of opsonins is the reason for inhibition of phagocytosis in this test, the results obtained could not possibly indicate "toxicity" of the antiseptics.

In a still later paper,[10] this test was modified to show actual destruction of leucocytes, instead of mere inhibition. The results are reported as "toxic activity," using human blood as the test tissue. The irreversible loss of phagocytic action after 10-minute exposure to the antiseptic is taken as an indication of cell death, and the results obtained are designated as "toxicity of antiseptics," despite the fact that leucocytes are not representative of body cells generally.

Another of the newer *in vivo* methods for testing antiseptics uses as tissue the chorio-allantoic membranes of eggs containing live embryos, and *M. pyogenes* var. *aureus* as the test organism.[11] The chorio-allantoic membranes of eggs containing embryos 12 days old are inoculated with a small quantity (0.02 ml.) of a dilute (1–10) suspension of broth culture of *M. pyogenes* var. *aureus*. Eighteen hours later and on each of the 5 following days, 0.2 ml. of the test antiseptic is dropped on the membrane. The day after the last treatment, the membrane is stroked with a cotton swab, then streaked on an agar plate. After incubation, the plates are counted for numbers of surviving organisms.

By this method, penicillin and the cationic detergents tested were found to be effective in killing the test organism, whereas iodine, phenol, and the organic mercurials employed were ineffective. It is inferred from these results that penicillin and the cationic detergents should possess therapeutic value under clinical conditions of use, whereas iodine, phenol, and the organic mercurials could not be clinically effective.

While this method of test simulates wound conditions more closely than do the other *in vivo* or *in vitro* tests previously suggested, it still fails to satisfy the conditions required of a satisfactory test procedure for antiseptics. The chorio-allantoic membrane of a fertile egg does not stimulate such human or animal tissues as skin or mucous membranes. Also, the amount of culture used in the test is too small to allow for a factor of safety. Still more important, the antiseptics tested must be applied in far greater dilution than is recommended for use in clinical practice. For example, iodine was used in dilutions of 1–2,500, 1–5,000, and 1–10,000, whereas 2 per cent and 7 per cent iodine are used in clinical practice. A 1–50 solution of iodine is the weakest dilution acceptable for general use. Finally, there is no provision in the method to prevent bacteriostatic activity of test antiseptics when the agar plates are swabbed from the treated membranes.

It is interesting to note that the antiseptics giving the best results by this test are those that are highly bacteriostatic against the test organism, *M. pyogenes* var. *aureus*. It could well be that their bacteriostatic effect on the agar plate accounts for the favorable results reported. On the other hand, iodine, phenol, and Azochloramide, in the dilutions used, are not highly bacteriostatic under the conditions of this test. Also, Metaphen and Merthiolate could hardly be expected to exert their effect in the dilutions used, namely, 1–5,000 to 1–30,000, when they are recommended for clinical use in 1–500 to 1–1,000 dilutions.

Here again, it is necessary to take a practical view of the problem. While it may be desirable to use a dilution of antiseptic that would not injure such delicate tissue as the chorio-allantoic membrane of fertile eggs, in human and animal practice it is far more important to kill the infective organisms. Infection must be controlled, even if the antiseptic does destroy some of the tissue cells. In actual clinical practice, antiseptics are used in concentrations that kill infective organisms, such as 1–50 phenol, 1–500 Metaphen, 1–1,000 Merthiolate, *etc.* Even though these concentrations of the antiseptics do destroy chorio-allantoic membrane, still they prevent infection by killing the invading bacteria, and at the same time they cause no serious damage to the human or animal tissues on which they are used.

It is, of course, desirable and even necessary to conduct a variety of tests on antiseptics in order to evaluate their possibilities for clinical use. This is the general consensus of bacteriologists and physicians actively interested in this field. Tests should be conducted to determine their various properties under a variety of conditions. One single test is not sufficient for this purpose, and, in fact, it is not expected that any one test will supply all of the information necessary to evaluate the properties of an antiseptic.

Recently, a very excellent suggestion has been made for the profile evaluation of germicides.[12] A combination of well-established tests is recommended for the purpose. Use of these tests is specified for determining the following properties of antiseptics: (1) the highest dilution of germicide capable of killing *M. pyogenes* var. *aureus*; (2) extent of bacteriostasis; (3) influence of organic matter; (4) speed of action; (5) penetrability; and (6) toxicity.

For this purpose, the following tests are employed: (1) modification of the F. D. A. test for determining killing dilution against *M. pyogenes* var. *aureus* (using standardized suspension); (2) bacteriostatic tests in 10 ml. and 100 ml. F. D. A. broth and thioglycollate medium; (3) similar tests in the presence of 10 per cent horse serum; (4) tests for killing, in time periods of from 1 to 10 minutes; (5) penetration tests, using the F.D.A. agar cup plate method, both with and without 10 per cent horse serum; and (6) toxicity tests on embryonic chicken heart tissue.

Studies of this character give considerable information relative to the properties of antiseptics. In fact, a combination of these tests will give information from which the practical value of an antiseptic may be predicted, or at least it will reflect its potentialities. Furthermore, the methods employed are all standard procedures that have been used separately for many years. It is now proposed that all of these tests be conducted as a panel. Since each test shows certain specific properties of antiseptics, collective results give a correct and useful profile of the antiseptics tested.

Recently, a serious effort to determine the germicidal spectrum of various kinds of antiseptics against a variety of test organisms was reported.[13] The method employed was a variation of the phenol coefficient type of test procedure, using a large number of representative pathogenic organisms. The purpose of the study was to determine the germicidal activity of representative germicides, including mercury compounds, phenol, cresol, hydrogen peroxide, alkali, acid, alcohol, silver nitrate, iodine, and quaternary ammonium compound. Fourteen microorganisms, of pathogenic or potentially pathogenic species, were used as test organisms. Cultures were grown in 2 per cent trypticase and, after exposure to various dilutions of antiseptics, were subcultured in U.S.P. liquid thioglycollate medium.

Results of these tests showed the wide variation in resistance of different species of the same test organisms and also between the various classes of bacteria. It was evident from results obtained that the resistance of test organisms must be determined before they are employed in laboratory tests on germicides. Also, it was shown that it is desirable to use a variety of such test cultures in order to obtain a full bactericidal spectrum. This test should, by all means, be included in studies of germicidal activity of antiseptics.

A review of recent developments in the field of antiseptics would not be complete without some reference to significant improvements in laboratory methods for testing fungicides and fungistats used in the treatment of fungus infections. Although such fungicides are not usually classed as antiseptics, they are drugs which are used on the human and animal body for the purpose of acting "against infection." This being the case, they fit the definition of antiseptics and should be classified similarly. Also, as most fungicides recommended for human use are also employed in the prevention of bacterial infections, they may well be considered in this connection. However, special methods of testing are required for fungicides. Higher fungi are employed as test organisms instead of bacteria.

One of the developments in this field during the past ten years is a laboratory method proposed for testing fungicides used in the treatment of epidermophytosis.[14] In this method, the five fungi most commonly found in athlete's foot infections are used as test organisms. Methods for determining both fungicidal and fungistatic activity are employed in the tests. The methods are severe and to some degree simulate clinical conditions of use. Fungicides and fungistatic products passing these tests have also been found clinically effective under practical use conditions.

Recently, the above fungistatic test methods have been studied from the standpoint of dose-response curves, and an "activity coefficient" has been determined.[15] Dosage-response curves are obtained by a modification of the agar cup plate technique. As a standard for comparison, a 5 mm. zone at 1 per cent concentration of the compound tested was arbitrarily chosen as the activity 1. Using this procedure, fungistatic agents can be compared on a numerical basis and be given numerical values. This constitutes a significant and valuable development in the field of fungistatic testing methods.

Relative fungicidal activity has also been determined by a modification of the Burlingame-Reddish method.[14] The modifications include use of fifteen-day-old fungus cultures, culture-fungicide contact of one minute, use of serum in the culture medium, and use of an acetone-water mixture for the washing phase of the test procedure.[16] By the use of this modification, it is possible accurately to classify fungicides by their minimal fungicidal concentration so that the relative merits of fungicidal compounds can readily be determined.

The accuracy of these methods in evaluating fungicidal and fungistatic activity has recently been demonstrated in a study of the antifungal properties of quinones and quinolines.[17] These studies demonstrate again the value of these methods for the purpose. They are highly recommended for use as standard methods for the evaluation and control of fungicidal and fungistatic products.

General Considerations

The evaluation of the merits of antiseptics and fungicides is not based entirely on results of laboratory tests. While methods of testing are useful in reflecting germicidal and bacteriostatic properties, the final criteria must depend upon actual effectiveness when used in practice. Under some conditions, however, the results of *in vitro* tests can be interpreted directly *after* information from tests under use conditions have been obtained.

In the case of phenol-like disinfectants—that is, disinfectants chemically related to phenol and which act against bacteria in a manner similar to phenol—this is comparatively simple. The use of the factor "twenty times phenol coefficient" is sufficient for calculating dilutions for use in practice. Other classes of compounds must be considered differently, and it is expected that the results of laboratory testing of these germicides may be interpreted more accurately as further information as to their practical value is obtained.

Hunter[18] emphasized this quite clearly in his discussion of the evaluation of antiseptics. He first warned against certain wide interpretations of results obtained by the Food and Drug Administration Methods of testing antiseptics. While these methods are used for determining germicidal and bacteriostatic properties, claims for effectiveness under conditions of use cannot be based solely on the results obtained by such laboratory tests. Hunter stated the situation quite clearly as follows: "The problem thus becomes one of fitting together the results of old established procedures with clinical observations and with the results of additional tests, some recently developed and some not yet conceived, to form a complete picture of the properties of a substance under scrutiny." It is evident that laboratory testing of antiseptics must be followed by practical *in vivo* tests, toxicity determinations, and by clinical tests under practical conditions of use for which such preparations are recommended.

The same is true of fungicides recommended for the treatment of dermatophytosis.[19] Here again, laboratory testing is simply the first step in the process of evaluating the efficacy of fungicides for therapeutic use; laboratory testing must be followed by clinical and toxicity tests.

The Council on Pharmacy and Chemistry of the American Medical Association[20] advised the following tests in support of any skin disinfectant submitted to the Council for consideration: *in vitro* tests in the absence and presence of serum; practical tests simulating conditions of use; *in vivo* tests by an animal method; tests for irritation and toxicity; clinical tests for efficacy and harmlessness; and tests on bacteriostatic activity as distinguished from germicidal activity of the skin disinfectant. It is clear that much more than *in vitro* tests are required for this purpose, not only in the case of skin antiseptics, but antiseptics generally, and fungicides as well.

There are certain other matters that must be considered in connection with the laboratory testing of germicides. For example, the nature of the test organism is of vital importance, not only the kinds of organisms used but their resistance as well. This feature of our commonly used methods has been the subject of extensive study over a period of many years.

As already stated, it is important that germicidal activity be determined on more than one test organism.[21, 22] Specifications for phenol resistance of representative test organisms have already been established.[21] Recently, Ostrolenk and Brewer[13] extended the list of suggested test organisms and reported the phenol resistance of representative strains. As emphasized by Brewer,[23] even the use of *M. pyogenes* var. *aureus* as the sole test organism does not provide the margin of safety so important in such tests, especially in determinations of germicidal activity of the synthetic detergents. In this connection, the high resistance of *Ps. aeruginosa* to germicides has been frequently noted. Many germicides which kill certain of the other test organisms will not kill *Ps. aeruginosa,* and this is further proof that the use of a single organism, even *M. pyogenes* var. *aureus,* is not desirable In fact, it is quite apparent that this resistant species (*Ps. aeruginosa*) should be used in all tests for antiseptic activity.

Recently, considerable interest has been shown in the testing of antiseptic soaps. Since *in vitro* tests have never been satisfactory for this purpose, practical *in vivo* tests are now being used. The one most commonly employed is that proposed by Price.[24] Since the Price method was developed for the study of the reduction of bacteria on the skin as the result of mechanical cleansing, it must be used with caution when employed in testing antiseptic soaps and synthetic detergents.

The method is one in which reductions of numbers of bacteria on the skin are determined by simple agar plate counts. Using surgical scrub-up technique, poured plate counts are made of the wash water at specified time periods. In the case of mechanical cleansing with soap and synthetic detergents, this procedure needs no special safeguards against bacteriostatic action in the agar plates. When antiseptic soaps and detergents are tested by this method, precautions are necessary to avoid false readings due to such bacteriostatic activity.

The following modification of the Price method is necessary in order to avoid such false readings: the arms, up to the elbows, are first scrubbed with a stiff brush in 2 liters of sterile water for 1 minute and 1, 0.5, and 0.1 ml. plated in poured nutrient agar plates immediately; the arms are then scrubbed with the antiseptic soap or antiseptic synthetic detergent in the manner described by Price, and then scrubbed for one minute in 2 liters of sterile water after the 1, 3, and 7 minute time periods and 1, 0.5, and 0.1 ml. plated in agar. Experience has shown that plating the wash water directly after each time period is satisfactory for non-medicated soaps and detergents, but, if this is done when antiseptic soaps and detergents are used, enough of the antiseptic may be transferred to the plates to be bacteriostatic even though the dilution is approximately 1–1,000 to 1–2,000 of the antiseptic employed. This is true especially of some of the new antiseptics now being used in antiseptic soaps and synthetic detergents.

Although this method of test is time-consuming, fairly accurate results are obtained which can be duplicated, especially if the above modification is employed. Furthermore, it is a practical test which duplicates actual use conditions. It is a satisfactory method for testing bacterial reductions on the skin following the use of either non-medicated or antiseptic soaps and synthetic detergents.

Discussion and Recommendations

The question naturally arises as to what use should be made of the various recently suggested methods of testing antiseptics. It is generally agreed that some of them should be employed in order to obtain more complete information as to the value of antiseptics than is possible with present *in vitro* methods. This does not mean, however, that *in vitro* tests should be discarded entirely, but that certain additional methods should be used to supplement the present test procedures.[25]

It is evident that the F. D. A. tests for antiseptics should not be discarded. They have been and are now accomplishing a useful purpose. Their use should be continued for the purposes for which they are intended. These purposes have been discussed, and it is apparent that the information obtained from these tests contributes materially to understanding of the germicidal and bacteriostatic activity of antiseptics.

Since there is little dissatisfaction with the present F. D. A. methods for testing antiseptic ointments, antiseptic dyes, *etc.* for bacteriostatic properties, our primary interest at this time is the testing of liquid antiseptics for germicidal activity. The vast majority of antiseptics used are liquid preparations which are claimed to be germicidal.

A panel of several different methods of test seems most desirable for determining the antiseptic properties of such drugs. The tests to be employed should vary with the recommendations made for each antiseptic. Tests for irritation and toxicity or destruction of body tissue must be included in such a panel. Based on the methods discussed above, the following panel of tests is suggested.

Panel of Methods for Testing Antiseptics

1. F. D. A. Method for Germicidal Activity, using *M. pyogenes* var. *aureus* at 37 C.[3]
2. F. D. A. Agar Cup Plate Method, with and without 20 per cent serum, for determining bacteriostatic activity and effect of organic matter.[3]
3. Infection-prevention method of Nungester and Kempf.[4]
4. Infection-prevention/Toxicity Index method as proposed by Spaulding and Bondi.[5]
5. Irritation tests on animals, such as instillation into the eyes of rabbits.
6. Tests for germicidal spectrum by the method suggested by Ostrolenk and Brewer.[18]
7. Oster and Golden[15] modification of the Burlingame-Reddish Method for determining fungistatic activity.

8. Golden and Oster[16] modification of the Burlingame-Reddish Method for determining fungicidal activity.
9. Modification of the Price[24] method for testing antiseptic soaps.

It is not suggested that all of these tests be applied to all antiseptics, but only those tests that are applicable to each preparation according to the purposes recommended or claims made. All of them, however, might well be used in research on new and even old preparations to determine more fully their usable properties. This panel represents a framework for research and control of antiseptics generally, including fungicides, which may be employed for reflecting the properties of this class of drugs.

Although no single test has yet been devised which would give all the information desired, use of the above panel will give useful information reflecting the antiseptic properties of these drugs and indicating their potential value under conditions of use.

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HALOGENS AND THEIR MODE OF ACTION

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The extensive use of various forms of chlorine and iodine as antiseptics has resulted in the accumulation of much information about efficiency of these halogens under various circumstances. There has been considerable study given to the factors affecting their germicidal activities and to the mechanisms of these effects.[1] Much less is known about bromine.[2, 3, 4]

The qualitative similarities and quantitative differences in many of the properties of chlorine, bromine, and iodine afford an excellent opportunity for observing the effects of certain isolated factors on germicidal activity. Accordingly, this paper will be concerned, for the most part, with the extent to which a few basic chemical properties of the halogens, such as hydrolysis, ionization, and formation of complexes can be used to explain quantitative differences in antiseptic power. The mechanism of the reactions leading to the death of the organism will not be discussed here. The interested reader is referred to another paper in this series and to a published report[5] for information on this subject.

One would expect that the rate of bactericidal action would be characteristic of the particular halogen compound present. Under usual conditions of use, however, one may have present, in addition to elemental halogen, hypohalous acid formed by hydrolysis, hypohalite ion from ionization of the acid, complex ions involving elemental halogen with halide ion, and N-halogen compounds. Furthermore, factors such as concentration, pH, and temperature should have quantitatively different effects on the germicidal activities of the three halogens because the equilibrium constants of the reactions involved are different.

Most of the work leading to quantitative conclusions has been carried out with bacterial spores, beginning with Levine and co-workers,[6] Meyers,[7] Tilley and Chapin,[8] and others. The advantages of using *B. metiens* spores, for example, have been pointed out by Charlton and Levine[9] and need not be enumerated here.

Accordingly, in FIGURE 1, we consider the comparative action on *B metiens* spores over a suitable concentration range of chlorine,[10] bromine, and iodine[11] at pH 7 and room temperatures. A solution of each halogen free of added halide was added to a buffered distilled water suspension of spores, extraneous organic matter being excluded as far as possible. The log of the time for 99 per cent kill is plotted against the log of the normality of the solution. Calculation shows that under these conditions chlorine exists chiefly as hypochlorous acid and bromine entirely as hypobromous acid, but iodine is present as molecular iodine. It is then not surprising that the usual order of halogen reactivities is not found and that iodine fails to show less germicidal power than bromine. It might be of some interest to note that on a weight concentration basis, iodine is considerably less active than chlorine, although still more active than bromine. Against an organism of low resistance these differences may become insignificant, as in the work of Beckwith and Moser.[12]

It is of interest from the theoretical point of view to compare the three halogens all as the hypophalous acids. FIGURE 2 contains the same type of comparative data, but, in this instance, all three halogens are in the form of hypophalous acids. The hypochlorous acid data were obtained at pH 6 and 7, and killing time is plotted against the calculated HOCl concentration for each point instead of against total active chlorine. The neglect of hypochlorite ion is justified, as will be shown later. Actually, the degree of ionization is not large, so that, for practical purposes, this curve is the same as in the previous figure. The data for hypobromous acid is exactly the same as in the previous figure, while the hypoiodous acid was especially prepared, free of iodide ion, and the test carried out at pH 6 for stability reasons. Hypoiodous acid is much less active than molecular iodine, so that we now find a regular decrease in germicidal activity as the molecular weight of the hypophalous acid increases.

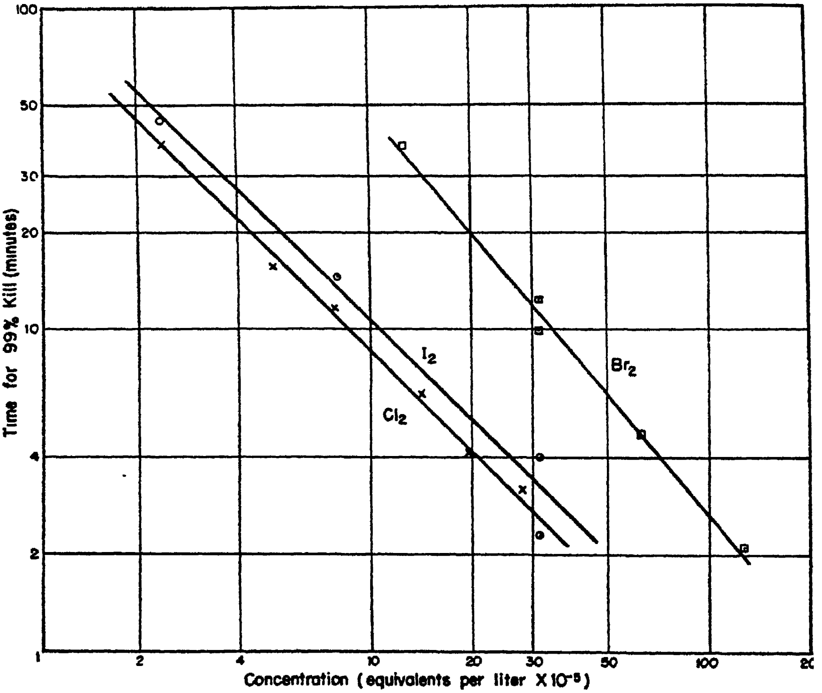

FIGURE 1. Effect of concentration on killing time Chlorine, bromine, and iodine at pH 7.0 and 25°C.

Further insight into the comparative germicidal activities of the three halogens themselves and into the relative importance of the several possible forms of each may be gained by following changes brought about by displacement of some of the equilibria involved. The hydrolysis of elemental halogen and ionization of hypophalous acid, involving hydrogen ion, are pH controlled so that pH effects can yield useful basic information. This is shown qualitatively in FIGURE 3, which compares the effects of pH on the action of chlorine,[10] bromine, and iodine[11] against *B. metiens* spores. The log of the time for 99 per cent kill is plotted against pH, in each case the concentration of active halogen being about 3×10^{-4} normal.

Differences in the hydrolysis and ionization constants bring about interesting differences and gradations in variation of germicidal power with pH. The rapid decrease of the activity of chlorine with increase of pH above 6 has been associated with the ionization of hypochlorous acid since the work of Andrews and Orton in 1904.[13] The first attempts to account for killing power solely on the basis of the undissociated hypochlorous acid were made by Holwerda[14] on coliform organisms and Helwig[15] on *B. metiens* spores. Chang[16] showed that the killing of the cysts of *Endamoeba histolytica* at various pH values in this range was solely a function of the concentration of this acid. We have shown that the killing time of *B. metiens* spores is the same logarithmic function of HOCl concentration, whether the latter is varied by changes in total active chlorine concentration at constant pH or by changes in pH at constant chlorine concentration.[10] Fair and co-

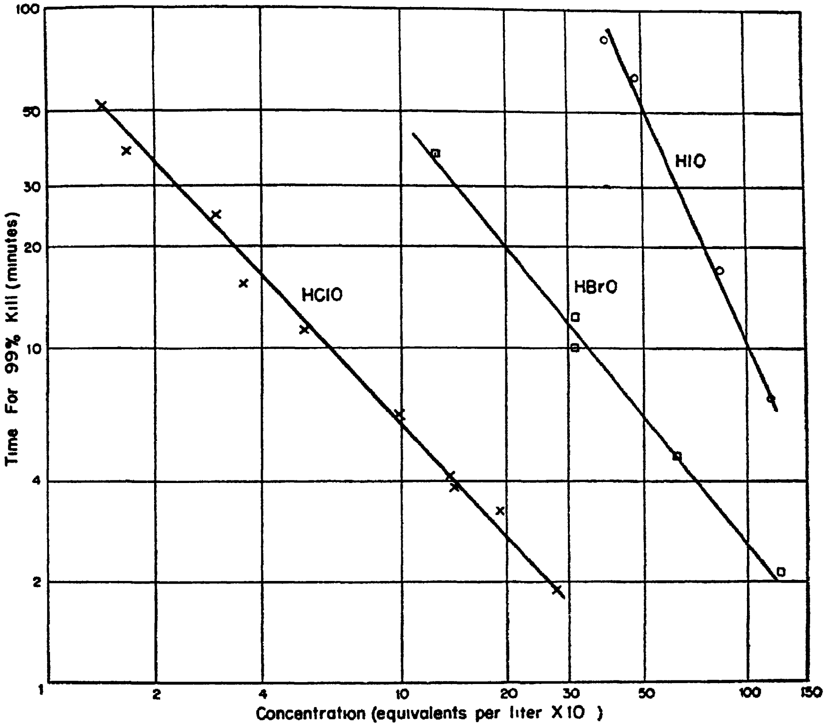

FIGURE 2 Effect of concentration on killing time at 25°C HClO, HBrO, and HIO.

workers[17] have demonstrated a similar quantitative relation for anthrax spores and have found a degree of quantitative agreement in the data of Butterfield *et al.*[18] on *E. coli.*

Additional experiments on *B. metiens* spores, in which the reaction is reduced to pH 2, show no further increase in activity beyond that at pH 6. Significant amounts of molecular chlorine would not be present in this range.[19] Chang[16] carried his studies on the cysts of *Endamoeba histolytica* down to pH 1, where 15 per cent of the chlorine is in the molecular form. The activity was slightly greater than that of hypochlorous acid.

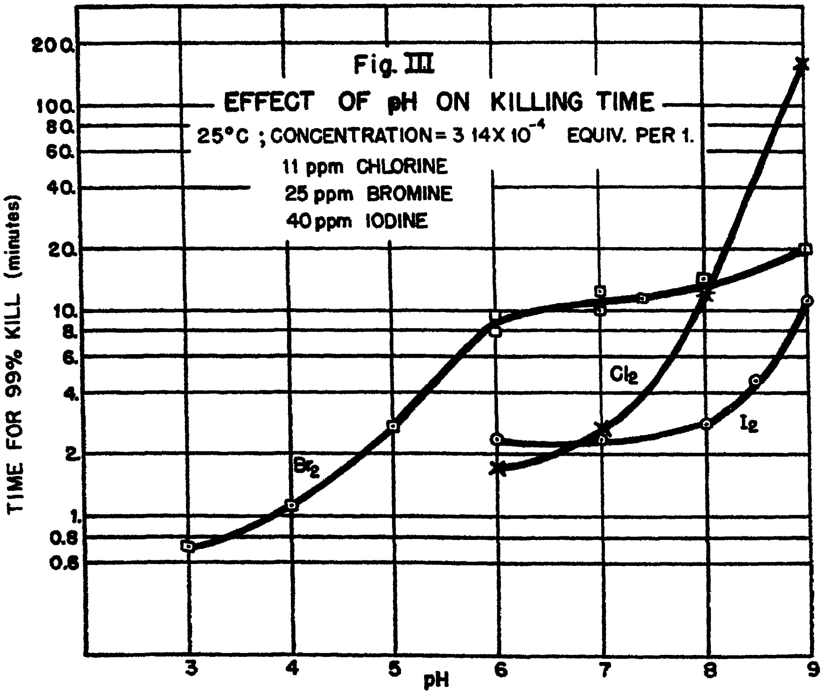

FIGURE 3. Effect of pH on killing time.

The region of constant activity of iodine coincides with the range where only molecular iodine exists, and the decrease in activity at higher pH values can be attributed to hydrolysis to the less active hypoiodous acid as shown by Wyss and Strandskov.[11] Gershenfeld and Fox[20] found a similar decrease with *E. typhosa.* It has not been possible to explain this quantitatively, using the relative degrees of activity of elemental iodine and hypoiodous acid. This disagreement may be due to the instability of hypoiodous acid, since similar studies with bromine yield more satisfactory results.

The bromine curve has two distinct parts, which reflect two different effects. The decrease in germicidal power from pH 4 to 6 corresponds to hydrolysis. In the region of approximately constant activity, molecular bromine is absent and the hypobromous acid concentration is constant. Finally, the decrease of activity in the higher pH range corresponds to ionization of this acid. In contrast to hypochlorous acid, we are unable quantitatively to account for the effect of ionization of hypobromous acid on germicidal activity, although the fact that ionization constant data for the latter are very contradictory may partially account for it. The data of Wyss and Stockton[4] for *B. subtilis* spores are in close agreement with these data for *B. metiens*.

TABLE 1

CALCULATED VALUES OF KILLING TIME FOR BROMINE AT VARIOUS pH VALUES (25°C: *B. metiens* SPORES; 3.14×10^{-4} EQUIVALENTS PER LITER BROMINE)

pH	C^*_{HOBr} (*equiv/L* × 10^{-5})	$C^*_{Br_2}$ (*equiv/L* × 10^{-5})	$t_{99\%}$ (*min.*)	
			Obs.	*Calc.*†
3	5.49	25.9	0.7	0.9
4	14.2	17.2	1.1	1.4
5	25.7	5.65	2.7	3.7
6	30.6	0.82	8.5	8.9
7	31.4	0.00	11	—

* Calculated from the hydrolysis constant 5.8×10^{-9}.

† Assuming that each equivalent of Br_2 equals 9 equivalents of HOBr and that the effects of the two are additive.

TABLE 2

KILLING OF *B. metiens* SPORES BY BROMINE IN THE PRESENCE OF KBr AT pH 7.0 AND 25°C (3.14×10^{-4} EQUIVALENTS PER LITER TOTAL BROMINE)

C_{KBr} (*mols/L* × 10^{-5})	C^*_{HOBr} (*equiv/L* × 10^{-5})	$C^*_{Br_2}$ (*equiv/L* × 10^{-5})	$t_{99\%}$ (*min.*)	
			Obs.	*Calc.*†
0	31.4	0	10.5	—
168	30.4	0.96	9.3	8.7
168	30.4	0.96	9.4	8.7
504	28.8	2.58	7.1	6.1
1510	24.9	6.54	4.0	3.3
4700	17.3	14.1	3.0	1.7
4700	17.3	14.1	2.4	1.7

* Calculated from the hydrolysis constant 5.8×10^{-9}.

† Assuming each equivalent of Br_2 equals nine equivalents of HOBr and that the effects of the two are additive.

On the other hand, there is rather good agreement between calculated and observed effects due to hydrolysis of bromine in the lower pH range. TABLE 1 compares theoretical and observed killing time due to various combinations of HOBr and Br_2 computed[21] to exist at the several pH values. In the computations, each equivalent of Br_2 was taken as being equal in killing power to nine equivalents of HOBr and a value for total effective HOBr obtained, the two activities being assumed to be additive.

The theoretical killing time was then calculated from the dilution curve for hypobromous acid at pH 7. The agreement between observed and calculated values would be even better with a higher value than 9 for the ratio of germicidal activities. The reason for the use of this value will now become evident, however.

A second convenient method of displacing the equilibria between the various halogen compounds is the addition of halide ion. At the concentrations used here, it is necessary to consider only two consequences of such additions, *i.e.*, suppression of hydrolysis and complex ion formation. In the case of chlorine it is not practical to study either effect.

Consideration of the bromine system shows that in the region of pH 7 reasonable amounts of added bromide will suppress bromine hydrolysis to a significant degree. Very large concentrations of bromide would be required to form tribromide[22] ion, so that there is opportunity here to study a single, uncomplicated effect. In TABLE 2 is shown the effect of several concentrations of bromide ion on the killing of *B. metiens* spores by 3.14×10^{-4} normal bromine at pH 7. As the bromide is increased, the germicidal power also increases due to conversion of hypobromous acid into bromine. In the second and third columns are given the concentrations of the two at equilibrium.[21] Again taking Br_2 to be nine times as active as HOBr, a theoretical killing time has been calculated exactly as above for the pH effect. The agreement with observed values is seen to be satisfactory. Thus, the ratio of molecular bromine to hypobromous acid has been changed by two entirely different and unrelated methods, with the same quantitative agreement in the effect on killing power.

TABLE 3

KILLING OF *B. metiens* SPORES BY I_2 IN EQUILIBRIUM WITH KI AT pH 7.0 AND 25°C

Dosage Iodine	*(equiv/L × 10^{-5}) KI*	*I_2 Concentration** *(equiv/L × 10^{-5})*	*99% Killing Time (minutes)*	
			Obs.	*Calc.*†
7.89	119	4.26	21	19
7.89	239	2.92	31	26
7.89	478	1.81	41	38
7.89	957	1.02	57	61
7.89	1910	0.551	96	99
31.6‡	0	30.2	2.3	3.9
31.6	6	29.0	4.3	4.1
31.6	24	25.2	5.1	4.6
31.6	241	11.9	9.5	8.3
31.6	1910	2.21	36	33
31.6	2410	1.81	39	38
31.6	24,100	0.158	220	272

* Calculated from the equilibrium constant 1.4×10^{-3}.
† Calculated from the equation $\log t = -2\ 25 - 0.81 \log C$.
‡ pH 6.0.

An entirely different reaction is to be expected in the iodine system. Addition of iodide ion to iodine solution at pH 7, where I_2 is the important constituent, converts a certain proportion of the latter to tri-iodide ion.

TABLE 3, taken from the data of Wyss and Strandskov,[11] shows how the killing power of two concentrations of iodine changes with increasing additions of iodide ion at pH 7. In contrast to the bromine experiment, germicidal activity is here greatly decreased by addition of halide ion, as Hailer had previously found on anthrax spores.[23] The middle column shows that this corresponds to the decrease in concentration of molecular I_2 by conversion to tri-iodide ion. It has been shown that the latter has relatively little activity. The killing time is the same logarithmic function of I_2 concentration, whether the latter is varied by using different dilutions of active iodine with a constant ratio of iodide or by changing iodide concentration with constant total active iodine. The last two columns show numerically how well the data follow such a dilution equation. Thus, quantitative differences in equilibrium characteristics account for directly opposite effects of added halide ion on the killing power of bromine and iodine.

TABLE 4
RELATIVE GERMICIDAL ACTIVITY OF N-HALOGEN COMPOUNDS (pH 7, 25°C)

Compound	Activity (*pure halogen* = *1*)
Monochloramine	1/36
N-chlorosuccinimide	1/13
N-chloropiperidine	1/300
N-chloro *p*-toluenesulfonamide	1/27,000
Monobromamine	1/2
N-bromosuccinimide	1/17
N-bromopiperidine	1/800
N-bromo *p*-toluenesulfonamide	1/11
N-iodosuccinimide	1/2
N-iododimethylhydantoin	1/2
N-iodophthalimide	1

N-halogen compounds are of interest from two points of view. In many applications of the halogens, nitrogen compounds are present which are capable of forming this type of compound. In other instances, the required characteristics of the antiseptic may be found in an N-halogen compound.

TABLE 4 shows the relative sporicidal activities of a series of N-chloro, N-bromo, and N-iodo compounds as determined by adding solutions of the compounds to the buffered spore suspension at pH 7. Activity is expressed as a fraction of that of the same concentration of free halogen at the same pH. The killing power of chlorine is greatly lowered in compounds of this type, not only with this organism but with many others.[8, 16, 24, 25] That of bromine is lowered just as much in some cases but to a lesser extent in others. Iodine is only slightly affected.

If the active agent is the hypohalous acid, as commonly believed,[26] germicidal activity should be related to the extent of hydrolysis. Actually the activity of most N-halogen compounds can be decreased by the addition of a large enough excess of the amine to repress hydrolysis.

The activity of the N-halogen molecule manifests itself when hydrolysis is sufficiently reduced. With some N-chloro compounds, we have accomplished this by adding excess amine to a point where further addition failed to change activity appreciably.[10]

When halogen is added to a medium containing nitrogen compounds, the extent and rate of formation of N-halogen derivatives determines activity. Bromine and chlorine in germicidal concentrations react with nitrogen compounds more rapidly than they kill spores. Consequently, their sporicidal power is practically the same as though they were allowed to react for a long time before contacting the spores. Iodine is very much less rapid in reaction with most nitrogen compounds. Its sporicidal power is not measurably altered by the presence of ammonia, *p*-toluenesulfonamide, and similar compounds.[11] High molecular weight nitrogen compounds, such as in peptone, decrease its activity. Gershenfeld and Witlin,[3] however, found iodine more active than chlorine or bromine when added directly to broth cultures of various organisms, although, in a simpler medium, chlorine is much more active than iodine on a weight concentration basis.

In summary, then, quantitative differences in the killing power of chlorine, bromine, and iodine can be accounted for in terms of the equilibria between the reaction products of a particular halogen with components of the environment, or, specifically, with water and amines. The killing rates of bacterial spores for the hypohalous acids and probably for the molecular halogens decrease in the order: chlorine, bromine, iodine. It is not known how well this would apply to other microbiological forms, even though this is the order of chemical reactivity in general. Knox *et al.*[5] have pointed out that spores may be killed by a mechanism that is entirely different from vegetative cells. The low killing power of the N-halogen molecule, compared to the hypohalous acid, is apparently quite general, the difference in chemical reactivities here being very great. Hypohalite ions and polyhalide ions are practically inactive against bacterial spores, and the former is just as inactive against other forms, such as *E. coli* at one extreme and the cysts of *Endamoeba histolytica* at the other. Probably, molecular halogen is more active than hypohalous acid in general. With bacterial spores, this is so for the three halogens; with the cysts of *Endamoeba histolytica*, it is so for chlorine; and, with *E. typhosa*, it is so for iodine.

We note the influence of a high degree of polarity in the inactivity of the ionic forms and the reduced activity of the hypohalous acids. This same influence has been observed in other instances, as, for example, for benzoic acid against both *E. coli*[27] and fungi.[28]

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IODINE AS AN ANTISEPTIC

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Since the latter part of the nineteenth century, much has been written extolling the alleged virtues of iodine as an antiseptic. This has been largely due to the fact that it fulfills a function that many bactericides do not and cannot fulfill. Iodine has been used in various forms as an antiseptic for the skin, wounds, and mucous surfaces of the body; for the sterilization of the air and of inanimate objects such as catgut and surgical instruments; as a prophylactic and therapeutic agent in diseases caused by bacteria, viruses, and fungi; for the disinfection of drinking water and swimming pool water; and for the sanitization of eating utensils. These uses of iodine will be considered later.

Properties of Iodine. Iodine, a solid crystalline element, bluish-black in color, with a bright metallic lustre, possesses the highest atomic weight of the common halogens. It is the only halogen which is solid at ordinary room temperature. Iodine has the unique property of changing spontaneously at room temperatures (20°C.) into the vapor state without first passing through a liquid phase. The vapor state is referred to as nascent iodine. True liquid iodine is rarely seen, except under special laboratory conditions.

Iodine is only slightly soluble in water (1:3450 at 20°C.) but freely soluble in aqueous solutions containing soluble iodides and also in many organic solvents. Iodine exhibits different colors when dissolved in organic solvents. Chloroform, carbon disulfide, carbon tetrachloride, and benzene solutions produce a violet color, whereas, in alcohol, ether, glycerin, and propylene glycol, a brown color is produced. The solubility of iodine has been reported to increase in the presence of cane sugar.[1]

Action of Iodine as a Bactericide. Iodine is a highly reactive element and, precisely because it is so reactive, it is a good germicide. When iodine acts as a disinfectant, free iodine is the effective agent.[2, 3] Sollmann[4] states, "Elementary iodine precipitates proteins, the iodine being partly absorbed, partly loosely bound and partly converted into iodide ions.... Since the iodine is loosely bound, it continues to penetrate so that the action extends deeply." McCulloch[5] believed that iodine destroyed microorganisms by the "formation of salts with proteins by direct halogenation."

The Bactericidal Efficiency of Iodine. In gathering laboratory data on the efficacy of an antiseptic one must recognize that today there is not as yet available a universal single laboratory test applicable under all conditions. In all instances, laboratory data should be obtained and interpreted under conditions paralleling as closely as possible those found in actual practice. In the final analysis, efficacy under conditions of use (as recommended in labeling) is the important criterion. When an antiseptic shows constantly satisfactory effectiveness by many different *in vitro* and *in vivo* methods, one can then state that it deserves a high rating of usefulness as a bactericidal agent.

Iodine has been used as a bactericidal agent for nearly a century, and there are many reports by different workers, including ourselves, as to its antisepticity. These reports have all indicated the high bactericidal efficiency and low tissue toxicity possessed by iodine. Discrepancies exist in the literature, however, as to the highest dilution of free iodine which is effective. This has been due to the use of different non-standardized strains of the same species of microorganisms, of different kinds of bacteria, and of different and varying techniques.

One of the most striking characteristics of iodine, especially noticeable to one who has studied the germicidal efficiencies of the medicinal dyes and the complex mercurials, is that the concentration of iodine necessary to disinfect does not vary greatly with different species of microorganisms.[6] We have checked and rechecked all of the techniques reported. It is our observation that the bactericidal efficiency of iodine solutions depends upon the concentration of free iodine, and that an increase in the amount of free iodine within a solution is indicated by an increase in the bactericidal efficiency.

Effect of pH. The pH values used in our experiments were kept within a range which theoretically gave no germicidal effect *per se* because of alkalinity (sodium hydroxide) or acidity (phosphoric acid). The buffer solutions used to keep the pH values constant did not act as a bactericide.

The reaction between iodine and sodium hydroxide formed sodium iodide, sodium hypoiodite, or sodium iodate.

$$I_2 + 2NaOH \rightarrow NaI + NaIO + H_2O$$
$$3I_2 + 6NaOH \rightarrow 5NaI + NaIO_3 + 3H_2O$$

As the alkalinity decreased (acidity increased), more free iodine was liberated. The starting point of this liberation of free iodine, although unknown, occurred at an alkaline pH. In acid solution, the reaction formed more free iodine.

$$NaI + NaIO + H_2SO_4 \rightarrow I_2 + Na_2SO_4 + H_2O$$
$$5NaI + NaIO_3 + 3H_2SO_4 \rightarrow 3I_2 + 3Na_2SO_4 + 3H_2O$$

There appeared to be a buffering action in 2 per cent free iodine solutions prepared from Tincture Iodine (USP XIII) or Iodine Solution (NF VIII) at the pH range of 6.0 to 7.2 with sterile F.D.A. broth as well as with 24 hour-old cultures of *S. aureus* in F.D.A. broth. Controls of 2.4 per cent sodium iodide solutions were prepared in buffer solutions. One ml. quantities of the 2.4 per cent sodium iodide solutions were added to varying quantities of sterile F.D.A. broth and the pH effect noted. The buffering action within the pH range of 6.0 to 7.2 was identical in the 2.4 per cent sodium iodide solution and in Iodine Solution (N.F. VIII), which consisted of 2 per cent iodine and 2.4 per cent sodium iodide. This indicated that the buffering action of 2 per cent aqueous iodine could be attributed to the 2.4 per cent sodium iodide.[7] The germicidal activity of iodine in the presence of excess potassium iodide does not vary with pH, since under such circumstances the hypoiodous acid (HOI) concentration is negligible even at pH 9.0.[8]

Color changes in 2 per cent iodine solutions started at pH 10.9 (10 ml. of N/1 sodium hydroxide + 100 ml. of 2 per cent iodine). The color, usually considered the end point in the chemical preparation of sodium hypoiodite, was attained at pH 11.6 (15 ml. of N/1 sodium hydroxide + 100 ml. of 2 per cent iodine solution). The solubility of iodine in 0.1 normal sodium hydroxide was found to be 1.694 per cent of iodine.[9]

The phenol coefficient of iodine at 20°C. with *Salmonella typhosa* varied from 5 at pH 9.55 to 20 at pH 4.93.[9]

From our findings published elsewhere,[7] it was evident that solutions containing free iodine, 2 per cent, displayed greater bactericidal efficiencies within one minute than were revealed by similar concentrations of free bromine or available chlorine under the conditions of the techniques used. Employing the same methods of testing, but adding McIlvaine's buffer solutions, 2 per cent iodine solutions were more effective antibacterial agents within a very wide pH range than were displayed by similar concentrations of the other halogens.[7]

Bactericidal efficiency tests with the dilute halogens (1:5000) revealed that free iodine solutions displayed more effective antibacterial activity against the test bacteria than did chlorine or bromine at 37°C. or 24°C. either in the presence or in the absence of organic matter. Iodine solutions of such strength were found to be effective for use as antiseptic washes and for irrigation purposes in pH ranges from 2.2 to 8.0.[10] We have evaluated iodine by the Klarmann-Wright technique[11] and have noted results comparable to those obtained by all other techniques.

Tests including other bactericides (halogens, phenols, mercurials [organic and inorganic], picric acid, the oxidizing agents, dyes, quaternary ammonium compounds, and others) reveal that the results obtained with iodine, employing the varying techniques, are comparable and that iodine deserves a high rating among the most efficient antiseptics in use today.

Bacteriostasis. Marshall and Hrenoff[12] made a clear differentiation between the properties of killing and inhibiting bacteria and reported that tincture of iodine killed in a 1:100 dilution and delayed the growth of microorganisms for 24 hours in concentrations between 1:100 and 1:1000. At dilutions of 1:1000 to 1:10,000, there was demonstrable inhibition, although growth was apparent within 24 hours. Sporulating bacteria were subject to bacteriostasis in the same manner as the nonsporulating species of organisms. However, the bacteriostatic effect varied with different organisms.

The technique of making transplants from clear subculture tubes has been employed to reveal whether the absence of growth was caused by bacteriostatic action.

Numerous organic and inorganic agents have been reported as being capable of neutralizing the effect of iodine.[13] Among the organic compounds used were serum, glycerin, syrup, feces, ascitic fluid, egg, milk, sputum, and urine. Among the inorganic substances were sodium thiosulfate, metallic mercury, and ammonia water. Sodium thioglycollate has also been added to transplant media, but, in the case of iodine, no differences in the results were obtained.[14, 15] Likewise, sodium thioglycollate, salts of the sulfites, and sodium thiosulfate have been incorporated into media as specific inactivating agents for any iodine which may have been carried over from medication mixtures. Sodium thiosulfate was usually considered because it could be obtained in pure form and in the same uniform strength each time. The amount of sodium thiosulfate required to react with iodine to produce sodium iodide, sodium tetrathionate, and water is based on the molecular equivalents of the chemicals, two molecules of thiosulfate for every molecule of iodine.

$$(2Na_2S_2O_3 \cdot 5H_2O + I_2 \rightarrow 2NaI + Na_2S_4O_6 + 10H_2O)$$

We have conducted investigations on iodine inactivation in which sodium thiosulfate was incorporated into media and compared with media to which thiosulfate had not been added. We have found that iodine enters into combination with the proteins present in nutrient broth. Because of its protein content, nutrient broth has the ability to inactivate the quantities of free iodine which may be carried over in all of the presently accepted bactericidal efficiency techniques[7]. The results of our studies with iodine inactivation have shown that the bacteriostatic and bactericidal dilutions are practically identical.

We have previously reported the intensity and rapidity of the action of iodine in experiments demonstrating the relationship between the killing and stasis of tissue *in vitro* by antiseptics in concentrations used in practice.[16] Here again, there is evidence that iodine, with a toxicity lower than phenol, kills immediately rather than by a prolonged period of stasis. This was later confirmed by Salle and Catlin,[17] who observed that the effective concentrations of iodine represented true killing values and that no bacteriostasis was involved. They also reported that the highest dilution of iodine killing within one minute (1:20,000) was the same as for ten minutes, a property not shared by any of the other antiseptics.

Since the effectiveness of an antiseptic may be altered in the presence of proteins or other organic matter, *in vitro* tests are frequently conducted in the presence of organic matter. This is usually accomplished by the addition of serum (human, horse, or other animal) before the addition of the broth culture of the test organism. The relationship of the interaction of iodine with blood and its constituents was reported by our laboratory in 1932,[18] and studied in greater detail by us in 1947.[19]

Toxicity Tests. Iodine has consistently been reported upon favorably in *in vitro* and *in vivo* toxicity tests. Tissue culture techniques have been used for the evaluation of iodine, employing human skin,[20] embryonic chick heart fragments,[21, 22, 23, 24, 25] frog tissue,[26] and rabbit spleen.[20] Penetration, injury, and skin sterilization have been conducted in *in vitro* and *in vivo* tests by many investigators on the skin of humans,[27] horses,[28] rabbits,[29] and other animals.[30] The effect of iodine has been studied on mucous membranes,[31, 32] root canals of the teeth,[33] and upon open wounds.[18, 33, 34, 35, 36]

Tincture of iodine has been used full strength on the human peritoneum.[37] The eye has been used for observation of ocular irritation and lysozyme inhibition.[38] One technique[39] combined germicidal efficiency tests, diffusion through paraloidion sac procedures, leucocytic toxicity determinations, and toxicity observations by the intradermal injection of the substances under test. Cutaneous lesions produced in mice with virulent staphylococci have been topically treated with antiseptics for evaluation purposes.[40]

Infection-prevention techniques employing mouse tails[41, 42] and abdominal tissue[31] have been devised as a means of determining the value of iodine and other substances as antiseptics in terms of their action on the skin as well as their effectiveness in preventing infection. A comparison of the percentage of mouse survivals and fatalities is used as the index of effectiveness.

An egg injection technique for toxicity was devised in our laboratory[43] and a comparison of the antiseptic's toxicity for chick embryos with its bactericidal efficiency determined.[43, 44] A modified technique of infecting the membranes of the embryo and then administering the antiseptic has been developed.[45, 46]

Manometric observations of the effect of iodine and other substances on microorganisms have been compared with mouse minimum lethal dosage of iodine.[47]

Iodine and other substances have also been evaluated by comparing bactericidal efficiency with the ability to destroy the opsonic complement contained in human and animal blood, as measured by the prevention of the white blood cells to engulf bacteria.[48]

In all of the *in vitro* and *in vivo* evaluations, iodine has been shown to be very high in germicidal efficiency and relatively low in toxicity.

Penetration. In 1930, Knaysi[49] presented an extensive review on penetration of the cell wall and the mode of action of bactericides, including iodine.

In 1932, Karns, Cretcher, and Beal[50] reported the penetration of iodine-iodide solutions in silk fabric and guinea pig skin. Biskine[51] demonstrated the rate of speed of penetration of aqueous iodine through frog skin. Nyiri and Jannitte[52] studied the action of iodine on the unbroken skin of dogs and rabbits and found that iodine, only in the form of its compounds such as potassium iodide, penetrated the live skin within 162 hours. Von Oettingen *et al.*[53] reported the penetration of iodine into leather. In 1933, Mallmann and Chandler[54] found that, of a group of recognized disinfectants, only colloidal iodine was able to penetrate and destroy the bacteria embedded in finely divided particles of avian fecal matter. Anderson and Mallmann[55] reported the superior penetrative power of iodine, using the wall of a chicken intestine, rabbit skin, coccidial oocysts, and strongylid larvae.

Sporicidal Efficiency. Iodine has been reported to possess sporicidal efficiency.[28, 56] The sporicidal action of iodine, at pH values ordinarily encountered, is due primarily to the free iodine, although, at high pH values, hypoiodous acid may also exert some action.[8]

Since 1902, catgut for surgical sutures has been reported as having been "sterilized" with iodine.[57] Because of its rapid and high bactericidal efficiency, iodine has been recommended as an "emergency sterilizing agent" for surgical instruments.[58]

Fungicidal Activity. Iodine has been found to possess both a high fungicidal and fungistatic efficiency against *Sclerotina fructicola*, *Alternaria solani*, *Penicillium expansum*, and *Rhizopus nigricans*.[59] Seltzer and Bender recently reported iodine as being fungistatic at 0.01 per cent and fungicidal at 0.1 per cent against *Monilia albicans*.[33] Iodine has been reported to possess fungicidal activity for *Trichophyton gypseum* and *Monilia albicans*,[60] *Epidermophyton inguinale*,[61] and various species of *Monilia*, *Torula*, *Epidermophyton*, *Trichophyton*, and *Saccharomyces*.[62] Strickler reported on the effective fungicidal properties of vaporized iodine in human skin diseases,[63] and iodine vapors have been claimed to destroy cultures of *Trichophyton tonsurans* within five minutes.[64]

Fruit has been wrapped in iodine-impregnated paper to retard the growth of mold responsible for destroying the fruit.[65]

Effect of Iodine on Viruses. Iodine has been demonstrated to possess virucidal activity. Tincture of Iodine U. S. P. XII, diluted to contain 0.1% free iodine, and Lugol's Solution U. S. P. XII, diluted to contain 1.0% free iodine, were effective in inactivating vaccinia virus CAEB within 3 minutes.[66]

Iodine has been used prophylactically with success against influenza[67] and herpes[68] and therapeutically in cases of variola and varicella.[69] It showed little activity, however, against the virus of tobacco mosaic.[70]

Kaiser[71] suspended various types of virus in water and aerated them with air which had first been passed through a 10 per cent alcoholic solution of iodine. By so doing, vaccinia virus was inactivated in thirty seconds and could not be reactivated. By suspending the virus directly in a series of dilute solutions of iodine, the minimum concentration of iodine for inactivation was found to be 1:1,000,000. Similar results were obtained with myxoma virus.

Disinfection of Air. In 1926, Lombardo[72] advocated the use of iodine as an aerial disinfectant. During an influenza epidemic, he diffused iodine vapor into the air of one classroom and, after fourteen days, found that there had been no cases of influenza in the iodine-treated room, whereas the number of cases was noticeable in the untreated classes.

In 1934[73] and in 1940,[74] iodine vapors were tested for their effect against bacteria at varying distances, and iodine was reported to head the list of 128 chemical substances tested for germicidal efficiency by this method. In 1944, iodine was reported to be effective as an aerial disinfectant at concentrations much below its saturation vapor pressure in air.[75] Iodine at a readily tolerable concentration of 0.1 mg./cu. ft. of air has been found to be an effective aerial disinfectant, rapidly killing freshly sprayed salivary organisms at humidities above 50 per cent.[76]

Disinfection of Water. In 1900, Malmejac[77] compared the water disinfecting properties of iodine with chlorine and bromine. He reported a reduction in organisms from 17,500 to 90 when purified with iodine, as compared to a reduction to 300 for chlorine and 190 for bromine.

Nesfield,[78] in 1905, recommended the use of iodine in concentrations of 1:260,000 and 1:130,000 for water disinfection. Two different tablets, a one two-grain tablet of iodide-iodate of soda and a one two-grain tablet of citric acid, were added to four gallons of water (1:130,000 free iodine) and, within one minute, the solution was capable of killing typhoid and cholera bacteria when present in a number over 50,000 per ml. The addition of similar tablets to one pint of water killed *B. anthracis* (vegetative) and *B. subtilis* (vegetative), present in numbers of 500 and 2000 respectively, in five minutes and was fatal to ordinary pyogenic bacteria.

Tanon introduced a process for "sterilization" of water by the use of iodine which was given considerable use by the French Armies during 1914 to 1918.[79] Another method, adopted by the French Army Sanitary Service, was the use of three compressed tablets (iodide, iodate, and tartaric acid) which, when added to water, liberated free iodine and effected "sterilization."

Many investigators have reported favorably upon the use of tincture of iodine for the "rapid sterilization" of water supplies.[80, 81, 82, 83] The U. S. Army, in 1922, demonstrated the efficiency of iodine as a water disinfectant and reported[84] that 5 ml. of 7 per cent tincture of iodine rendered a Lister bagful (36 gallons) of raw Potomac River water safe for drinking within 30 minutes. This is approximately equivalent to one drop of 7 per cent tincture of iodine per quart of water.

In 1940, the U. S. Public Health Service recommended[85] the disinfection of drinking water by the addition of one teaspoonful of tincture of iodine to one barrel of water (about 55 or 60 gallons), stirring, and allowing to stand for one-half hour before using. Eight drops of tincture of iodine 7 per cent sterilized one quart of water in five minutes. Rosenau, in 1941, reported that iodine in concentrations of 1:1,000,000 to 1:30,000 instantly killed the streptococci present in water which had been responsible for paralysis and death when administered to rabbits.[86]

In 1947, a U. S. patent was issued to Marks and Strandskov,[87] in which they claimed better results were obtained with iodine than with chorine in the sterilization of swimming pool water, since the "sterilization rate" of iodine was less effected by ammonia. The patented process continuously subjects swimming pool water to the introduction of chlorine in sufficient amount to maintain a slight chlorine residual. Simultaneously, iodine (a halogen of greater atomic weight) in the form of sodium or potassium iodide is introduced to maintain a desired concentration of iodine atoms in the pool. The chlorine oxidizes a substantial part of the iodide to free iodine. Available chlorine may be used in the form of chloramines; in fact, where an iodide has been introduced, the oxidation of iodide to iodine occurs, provided sufficient chloramine or other available chlorine is present to afford a quantitative reaction. According to the patent, this provides a more rapid, more prolonged, and more intense sterilizing effect than is possible by the use of chlorine alone.

In 1948, free iodine was reported to destroy, within one minute, all *Leptospira* in water containing 3,000,000 organisms/mg. when the residual iodine was between 0.5 and 2.0 parts per million.[88]

On the basis of comparative tests, we have evaluated the efficiency of iodine in swimming pool water.[89] For the disinfection of water we have found 0.1 p.p.m. to 0.25 p.p.m. of free iodine to reduce considerably the commonly encountered vegetative organisms. Complete destruction of these organisms in the water resulted, at times, within 1 minute at 0.5 parts per million of free iodine, often within the range of 0.5 p.p.m., and always at concentrations of 1 p.p.m. or more.

Sanitization by Iodine. Employing food utensil sanitization procedures, we have found that iodine satisfies the requirements set forth by the U. S. Public Health Service's "Ordinance and Code Regulating Eating and Drinking Establishments" (1943). Free iodine in concentrations of 50 p.p.m and above killed *E. coli* within 20 seconds. As a safety factor, we recommend the use of 200 p.p.m. free iodine for eating-utensil sanitization either in the sick room or for routine use.[89]

In a report to be presented shortly, we have found 2 per cent to be rapidly effective in disinfecting clinical thermometers.

Preparations Containing Free Iodine. Iodine Tincture U.S.P. (containing 2 per cent free iodine) is the preparation most frequently used as an antiseptic. Iodine Solution N.F. (containing 2 per cent free iodine), an aqueous solution, can be employed effectively to replace the tincture. We have in the process of publication a paper detailing the efficiency of Iodine Solution containing varying concentrations of propylene glycol.

There are various inorganic and organic mixtures, available as powders or tablets, which release iodine upon contact with water and are being used in practice as antiseptics. The diglycine hydriodide tablet used by the military for the disinfection of small quantities of water is one of the organic type widely used. Recently, a mixture of powders was presented as a new bactericidal agent for infected wounds, the efficiency of which depends upon the release of free iodine and the emission of a small amount of ultraviolet light.[90] Occasionally, ointments containing free iodine are used.

Potentiation. The potentiation of iodine's bactericidal efficiency has been reported with the use of wetting agents.[91] A doubtful and irregular increase in the phenol coefficient from 3.7 and 4.3 to 4.3 and 5.5 resulted from the addition of 0.1 per cent Aerosol OT. On the other hand, Salle[92] demonstrated that, by balancing an oxidation-reduction system in the presence of iodine, he was able to raise the killing dilution of iodine from 1:20,000 to 1:450,000. This potentiation was accomplished with manganous and ferric chloride.

The efficiency of iodine was reported to be increased in combination with urea.[93] Iodine in the form of soluble iodide has been reported to produce a synergistic effect *in vivo* in combination with antibiotics.[94, 95]

Conclusions. Over three decades' experience in testing iodine and other antiseptics by all of the recommended and commonly used techniques and by those devised in our laboratory has provided us with data upon which to base the following conclusions:

(1) Iodine is a highly effective bactericidal agent. It does not vary appreciably in its effectiveness against many different species of microorganisms. One ml. of 2 per cent iodine is effective within one minute against 20 ml. of a 24 hour culture of *S. aureus*, *S. typhosa*, *E. coli*, and *P. aeruginosa*.

(2) The bacteriostatic and bactericidal dilutions are practically identical.

(3) It is not necessary to use sodium thiosulfate or other chemicals as inactivating agents when performing the commonly employed bactericidal efficiency tests. Iodine is inactivated by the culture media used as transplant media in these techniques, since the protein contained therein acts as the inactivating substance.

(4) The highest dilution of iodine (1:20,000) killing within one minute is the same as for ten minutes, a property not shared with any of the other antiseptics.

(5) Iodine combines with blood and blood fractions to form complex organic substances containing antibacterial activity.

(6) Iodine possesses a very low tissue toxicity as determined by the many varied and different *in vitro* and *in vivo* techniques.

Two per cent tincture or aqueous solutions of iodine is especially useful as a preoperative skin antiseptic and for the treatment of wounds and various skin affections. Clinical thermometers can be quickly disinfected by 2 per cent tincture or aqueous solution.

(7) Solutions of free iodine 1:5000 (0.02 per cent) are effective for use as an antiseptic wash or for irrigations in pH ranges from 2.2 to 8.0. Such solutions have wide use in surgery.

(8) For the disinfection of water, we have found 0.1 p.p.m. to 0.25 p.p.m. of free iodine to reduce considerably the numbers of the commonly encountered vegetative organisms. Complete destruction of these organisms in the water occurs at times within 1 minute at 0.5 parts per million of free iodine, often within the range of 0.5 p.p.m. to 1.0 p.p.m., and always at concentration of 1 p.p.m. or more.

(9) Employing food utensil sanitization procedures, we have found that iodine satisfies the requirements set forth by the U. S. Public Health Service's "Ordinance and Code Regulating Eating and Drinking Establishments" (1943). Free iodine in concentrations of 50 p.p.m. and above killed *E. coli* within 20 seconds. As a safety factor, we recommend the use of 200 p.p.m. free iodine for eating-utensil sanitization either in the sick room or for routine use.

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BACTERIAL RESISTANCE

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Resistance is seldom an "all or none" phenomenon. The concentrations of antiseptic required to inhibit the various species of bacteria, the strains within a species, or the individuals within a strain usually can be represented as a continuous function, with all intermediate values represented and distributed normally. In killing studies with bacteria, similar variations occur, but those which exist between individuals are more difficult to study. The demands of practical problems in chemotherapy have stimulated work on resistance phenomena, and considerable information on the resistance to bactericidal substances can be obtained by analogy to these studies. As with bacteriostatic substances, organisms with a heritable resistance to killing arise as occasional mutants in the population. The mutation to resistance has been so widely discussed[1, 2] that it requires no further elaboration here. Although the data are not complete, evidence has been presented[3] to suggest that, at least with some chemical agents, when there are wide differences between the resistances encountered in various species, there will also be wide differences in the strains and the individuals within strains. The converse is probably true in part also; that is, if with a new drug there is wide variation in resistance among the individuals in a test culture, the strains within that species also exhibit a relatively wide distribution in resistance to the drug.

The relationship between resistance of strains and of individuals within a strain is negated to some extent by the selective action exhibited by culture media. The standardization of the culture media that are used by microbiologists has been a necessary step for securing analogous results in test experiments, but it has tended to mask real variations in organisms as they exist in nature; that is, our stock cultures are more nearly uniform than their source strains. Although we refer to our stock cultures which have not been subjected to mutation procedures as "wild type," there are good reasons for believing that the truly wild type strain exhibits varying resistance depending on the selective action of its past environment.

Most organisms require a period of adaptation to a new culture medium. As we study adaptations, we find an increasing number of examples of this adaptation being a selection of pre-existing variants or mutants in the population, or, as Fildes[4] suggests, "training bacteria . . . may be looked upon as a cumbersome method for selecting genetic mutants." While we generally design experiments to select organisms that differ from their fellows in one single respect in order to simplify the study of the inheritance phenomenon, population shifts that normally occur in cultures of microorganisms developing under different conditions are extremely complex. Slight changes in the media or conditions of culture may promote the development of a strain which has characteristics that do not, with our present understanding of the subject, exhibit a simple cause-effect relationship. For example, TABLE 1 shows what occurs when *Micrococcus pyogenes* var. *aureus* is grown in a chemically defined medium deficient in methionine. Such a medium appears to favor the development of the streptomycin-resistant mutants in the population, and, at the time of sampling, it is evident that they had accumulated to a considerable degree as compared with the control. If the organisms are cultured at temperature or pH values other than those customarily employed, immediately the population changes, and the resulting population exhibits a considerable, and generally unpredictable, variation from that exhibited under so-called "normal" conditions.

It is easy to measure the resistance distribution with many bacteriostatic substances, since the organisms which form colonies in the presence of varying amounts of the inhibiting agent can be counted readily. The detection of mutants resistant to killing agents is more difficult. Most bactericidal substances destroy organisms after a more or less logarithmic pattern. The most resistant organisms in a population are not generally the chance survivors after a killing experiment. This is shown in FIGURE 1, where curves are plotted to show death rates of a resistant and wild-type strain. If a few organisms of relatively high resistance were present in the sensitive population, they would not be detected among the survivors. The dotted line parallel to the killing curve of the resistant population shows that even if the resistant mutants made up 0.1 per cent of the original culture, they would have been eliminated after 5 minutes' exposure and none would have been found among the last survivors.

TABLE 1

EFFECT OF GROWTH IN SYNTHETIC MEDIUM DEFICIENT IN METHIONINE ON THE NUMBER OF STREPTOMYCIN-RESISTANT MUTANTS OCCURRING IN CULTURES OF *M. pyogenes* VAR. *aureus*

Experiment	*Methionine concentration*	*Mutants per million**	
		Penicillin resistant	*Streptomycin resistant*
1	M/50,000	30	24
	0	25	119
2	M/50,000	47	141
	0	34	1,201
3	M/50,000	88	33
	M/500,000	58	68
	0	68	124

* Mutants assayed on nutrient agar containing either 0.05 units per ml. penicillin or 5 units per ml. streptomycin.

With a number of agents, an estimate of the organisms resistant to killing is possible because they also exhibit increased resistance to the bacteriostatic action of the same chemical. This permits an assay of the inherited resistance in the population and also facilitates the selection of a strain resistant to the killing action of the chemical. The ratio of bactericidal to bacteriostatic concentration does not remain constant with the substrains. Nevertheless, crude though it may be, an estimate of the number of resistant organisms which exists in a population can be obtained. TABLE 2 shows the effect of variation of media and temperature of incubation on the production of strains resistant to the bacteriostatic action of phenol. Growing a test culture for the phenol coefficient procedure in a casein hydrolysate broth or at a slightly elevated temperature will make the culture more resistant to the killing action of phenol. From this table, it is evident that these procedures select in favor of the resistant mutants, and further ex-

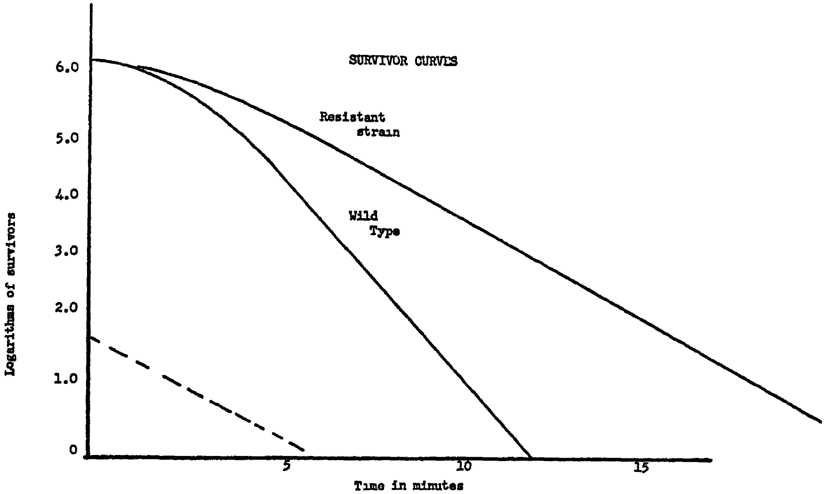

FIGURE 1. Killing curves of bacteria. The dotted line paralleling the upper curve shows why a few organisms from that strain, when mixed with the sensitive strain, are not found in the survivors.

TABLE 2

NUMBERS OF PHENOL RESISTANT ORGANISMS IN CULTURES AS AFFECTED BY TEMPERATURE AND MEDIUM

Phenol concentration	*Cultures**		
	Nutrient broth		*Casein hydrolysate broth*
	37°C.	*40°C.*	*37°C.*
0.00	1,020,000	1,000.000	1,000.000
0.10	250,000	150,000	200,000
0.11	40,000	65,000	50 000
0.12	12	600	800
0.13	0	130	200
0.15	0	10	50

* *Micrococcus pyogenes* var *aureus* grown as indicated for 8 hours and then assayed at 37 C. in nutrient agar containing the indicated concentrations of phenol to determine the resistance distribution.

periments showed that, on subsequent transfers, under the same conditions of temperature or medium constituents, a high percentage of the population would become resistant to the bacteriostatic action. This was reflected in the killing curves and in phenol coefficient tests.

The selection of mutants is not the only effect of the culture medium on resistance of organisms to germicidal action. Often the materials carried over from the medium to the test medication tube affect the result. Sometimes the entire population acquires a resistance that is not inherited. For example, the growth of organisms in a medium rich in phospholipids will result in a population with a higher resistance to surface active agents.

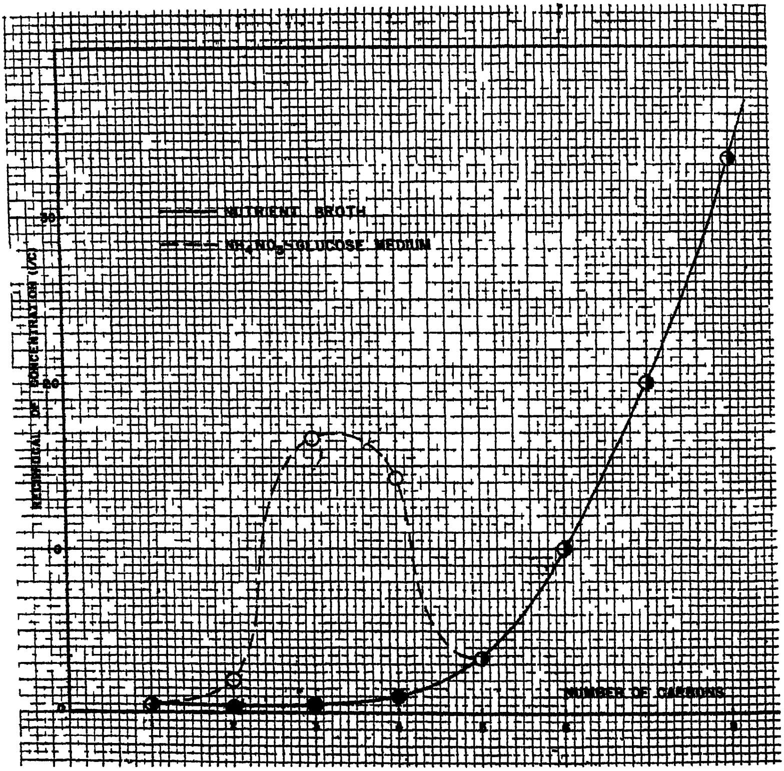

FIGURE 2 Effect of type of medium on concentration of fat acids of varying chain length required to inhibit *E coli*.

Sometimes a resistance is developed that is apparent in one test medium but not in another. FIGURE 2 shows the inhibitory concentrations of fat acids of various chain lengths, in both synthetic and nutrient broth. In nutrient broth, activity increases with chain length, as has been demonstrated by various workers. In a synthetic medium, the 2, 3, and 4 carbon acids show an additional activity which they do not show in nutrient broth. This can be ascribed to a competitive inhibition with certain medium constituents involved in the synthesis of pantothenic acid. In the complete medium, adequate pantothenic acid as well as precursors are present, and no additional inhibition results. We have developed a strain resistant to the enhanced action of the short chain fat acids. This strain showed no increase in resistance against the long chain or against any other fat acid when tested in nutrient broth. Nor was it more resistant than the wild type to the killing action of the short chain fat acids when tested in buffer. The resistance had in some manner surmounted the block in the pantothenate synthesis but had not modified the sensitivity to the surface activity of these compounds. On the other hand, we have developed another strain slightly more resistant than the parent to long chain fat acids. This strain is slightly more resistant to all the fat acids than any of the wild type when tested on nutrient broth.

When the wild type of a microorganism is sensitive to an antimicrobial agent, strains derived by selecting resistant mutants may owe their resistance to any one of a variety of mechanisms. Sometimes the mechanism or mechanisms may serve to interfere with several inhibitors and "cross resistance" is observed.

(1) Ehrlich[4] observed that the trypanosome strains which became resistant to the killing action of basic dyes were able to prevent the dyes from penetrating through the membrane and staining the protoplasm. Spirochaetes that are resistant to arsenicals do not absorb the arsenicals into the cell as do the sensitive strains. A fluoride-resistant strain of propionic acid bacteria has been reported[5] which is resistant by virtue of the failure of fluoride to penetrate to the active site. In general, however, the "loss of permeability to the inhibitor" has not been as effective in explaining acquired resistance of bacteria as it has been with spirochaetes and trypanosomes. Peacock and Hinshelwood[6] report that dye-resistant cells absorb as much or more dye than the parent strain and regard this as definite evidence against the permeability theory for their inhibitors. Maas and Johnson[7] found no striking differences between absorption of radioactive penicillin by resistant and sensitive staphylococci.

(2) Some, but not all, sulfonamide-resistant strains produce increased amounts of the competing metabolite, *p*-aminobenzoic acid. In an extreme case with a *Neurospora* mutant which requires sulfanilamide for growth, it has been suggested[8] that the organism produced toxic quantities of *p*-aminobenzoic acid, so that only the inhibiting action of sulfanilamide permits growth. Many strains of streptomycin-resistant bacteria are also streptomycin-dependent, which suggests another sample of an over-compensation by the organism that can be controlled only by the presence of the inhibitor.

(3) Some organisms can destroy the inhibitor. A few penicillin-resistant strains are resistant by virtue of possessing the enzyme penicillinase. A similar situation exists with pyrithiamin, an analogue of thiamin, in which the thiazole portion of the molecule is placed by pyridine. A strain became resistant to this substance by developing an enzyme which split the pyrithiamin molecule into two nontoxic parts. The tubercle bacillus, a Gram-

positive organism which exhibits an anomalous resistance to crystal violet, has been reported to destroy the crystal violet or at least to produce clear zones around colonies growing on crystal violet agar.

(4) Since penicillin inhibits the transfer of certain amino acids through the membrane, extreme resistance to penicillin should result when organisms are able to synthesize the necessary amino acids in the cell. This happens with at least one strain of *Micrococcus pyogenes* var. *aureus*; a resistant mutant[9] has acquired the ability to grow without an exogenous supply of amino acids. Conversely, it has been reported that biochemically deficient mutants of some organisms are more sensitive than the parent strains.[10]

(5) By increased production of the inhibited enzyme, the organism would have enough enzyme to unite with the inhibitor and still have some left over for action with the metabolite. One theory on adaptive enzymes predicts increased production of such enzymes in the presence of inhibitors. Cells grown at a pH too acid for the optimum activity of urease and catalase will produce more of those enzymes and thus compensate for the lessened activity. This may be the overcompensation suggested above. Morse and Carter[11] report that an irradiation-resistant strain of *E. coli*, also resistant to nitrogen mustard and H_2O_2, contains 3 to 4 times as much desoxyribonucleic acid as the strain from which it was derived. Perhaps this strain has double chromosomes or nuclei. We have some evidence that such doubling of genetic material accounts for the resistance of some of our strains that are resistant to killing by heat and chemicals.

(6) The organism may utilize an alternative pathway to bypass the inhibited function. The streptomycin-resistant *E. coli* studied by Umbreit[12] apparently do not utilize the citric acid cycle.

(7) Cytological variation may contribute to resistance to disinfectants. Capsules are known to protect organisms from the action of lethal agents; large cells are more resistant than small cells. Gram-negative bacteria have near their surface a phospholipid of the cephalin type, which appears to be associated with resistance to some surface-active agents. Increase in cell size appears to accompany resistance to a variety of agents.

The question arises as to the possible application of research on microbial resistance. What can we do about it? A knowledge of those factors which tend to increase the mutation rate and to select in favor of the spontaneously occurring mutants resistant to chemicals should be of assistance in some practical problems. Once resistance to an antimicrobial agent is established, it is logical to use a different type of agent, for which no cross resistance exists. There are occasional bits of evidence to suggest that an attempt to modify the organism to make it susceptible to the lethal agent might eventually lead to success. When a microbe is resistant by virtue of an enzyme system which bypasses the inhibited function, the synergistic action of two drugs may result in success. Several examples of this have been presented. Certain pterin analogues together with sulfonamide will inhibit a microbial population resistant to considerable amounts of either drug.

A number of workers have attempted to sensitize resistant organisms by a biological procedure. Voureka[13] found that a penicillin-resistant staphylococcus will become more sensitive when grown in association with a sensitive streptococcus. Winner[14] confirmed this and found in addition that the resistance of the streptococcus increased. George and Pondalai[15] were able to modify the resistance of organisms by cell extracts. Certain aspects of this problem have been studied in our laboratory in connection with our work on mutations and sexuality in bacteria. TABLE 3 shows an experiment with a stock strain of *Escherichia coli* and a sulfonamide-resistant strain developed from it. Nucleoprotein extracts were prepared from each organism and added to growing cultures. It is evident from the table that the extract modified the resistance of some of the individuals of the culture. This modified resistance persisted in subcultures and may be analogous to transmutation experiments now reported for several species. Such experiments have not been uniformly successful. Repeated failures have resulted from attempts to modify the antibiotic resistance of *M. pyogenes* by nucleoproteins and nucleic acids prepared by a variety of procedures and many sources. Certainly, the occasional success obtained by this method of modifying resistance suggests that our attention should not be centered completely on the search for new and better antibacterial agents for use against resistant organisms. It is not inconceivable that we might alter the organisms to fit the presently available drugs.

TABLE 3
TRANSMUTATION IN *E. coli*

Stain	*Nucleoprotein* in growth medium*	*Colonies appearing on assay agar containing sulfanilamide*				
		0	*2*	*5*	*10*	*30 mg.%*
Wild	None	130M	2M	900	0	0
Wild	Wild	125M	3M	700	0	0
Wild	Resistant	120M	7M	20T	100	10
Resistant	None	140M	112M	8M	800T	0
Resistant	Wild	140M	100M	420T	2T	0

* Nucleoprotein prepared by lysing cultures in 0.1 per cent desoxycholate at 50°C. and precipitating nucleoprotein from supernatant with alcohol.

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THE RELATIONSHIP OF CONCENTRATION AND GERMICIDAL EFFICIENCY OF ETHYL ALCOHOL

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Alcohol is perhaps the most widely used substance for a disinfecting action on the skin prior to hypodermic injections, venipunctures, finger pricks to obtain blood for blood counts and hemoglobin determinations, and before other procedures which break the intact skin. In spite of this wide usage, textbooks on bacteriology, in general, state that 95 per cent ethyl alcohol is not a good disinfectant, but that the germicidal action is increased if the concentration is reduced to 60 to 70 per cent. The widespread use of 95 per cent ethyl alcohol as a disinfectant would not seem justified if the impressions gained from the statements in the textbooks are true. Another important aspect of the germicidal action of ethyl alcohol is the frequent use of solutions of approximately 50 per cent as a vehicle for many proprietary preparations marketed as skin disinfectants. The germicidal action of ethyl alcohol in varying concentrations has been reinvestigated.

Very few textbooks give reference to the experimental data upon which are based the statements that 95 per cent ethyl alcohol is practically worthless as a disinfectant. Apparently these statements, which have been perpetuated in the textbooks for decades, are based on experimental work reported before or at about the beginning of the present century. In those early days of bacteriology, it was the customary procedure to test germicides against microorganisms which had been dried on threads or garnets.

At the present time, the customary procedure for determining *in vitro* bactericidal action is to add 0.5 ml. of an actively growing culture of a microorganism to 5 ml. of the disinfectant solution being tested. In order not to alter the concentrations of the alcoholic solutions being tested, 0.5 ml. portions of the culture under test were placed in sterile test tubes, centrifuged, the supernate discarded, and 5 ml. of the alcoholic solution being tested were mixed with the sedimented cells. This mixing was thorough and practically instantaneous, as the disinfectant solution was added forcefully from a 5 ml. pipette and the culture-germicide mixture alternately drawn into and expelled from the pipette. Because of the low surface tension of solutions of alcohol, it was a difficult and uncertain procedure to subculture the culture-germicide mixture with the usual bacteriological platinum loop. Instead, one drop of the culture-germicide mixture was transferred by means of a pipette to the subculturing medium. When subcultures were made at intervals of a few seconds, a drop from the same pipetteful of culture-germicide mixture was dropped into separate tubes of subculturing medium at the proper time intervals. Using the same pipette for discharging several drops tended to minimize any error due to variation in size of the drops. The subculture medium was extract broth. For the hemolytic streptococci, the extract broth also contained 10 per cent sterile normal horse blood. The tests were carried out at room temperature, but the temperature of incubation was 37°C. The nature of the microorganisms growing in the subcultures after 3 to 4 days was verified by microscopic methods. The absolute ethyl alcohol employed met the United States Pharmacopoeial requirements for absolute alcohol. The solutions of various concentrations of ethyl alcohol were prepared by diluting absolute

TABLE 1
THE EFFECT OF VARIOUS CONCENTRATIONS OF ETHYL ALCOHOL
TEST ORGANISM: *Serratia marcescens*

Alcohol (C_2H_5OH)		*Exposure of test organism to germicide*												
		Seconds					*Minutes*							
% by vol.	*% by wt.*	10	20	30	40	50	1	2	3	4	5	10	15	20
100	100	−	−	−	−	−	−	−	−	−	−			
95	92	−	−	−	−	−	−	−	−	−	−			
90	85	−	−	−	−	−	−	−	−	−	−			
80	73	−	−	−	−	−	−	−	−	−	−			
70	62	−	−	−	−	−	−	−	−	−	−			
60	52	−	−	−	−	−	−	−	−	−	−			
50	42	−	−	−	−	−	−	−	−	−	−			
40	33	−	−	−	−	−	−	−	−	−	−	−	−	
30	24	+	+	+	+	+	±	−	−	−	−	−	−	−
25	20			+	+	+	+	+	−	−	−	−	−	−
20	16						+	+	+	±	+	+	−	−
Dist. water (control)							+	+	+	+	+	+	+	+

TABLE 2
THE EFFECT OF VARIOUS CONCENTRATIONS OF ETHYL ALCOHOL
TEST ORGANISM: *Pseudomonas aeruginosa*

Alcohol (C_2H_5OH)		*Exposure of test organism to germicide*													
		Seconds					*Minutes*								
% by vol.	*% by wt.*	10	20	30	40	50	1	2	3	4	5	10	15	25	30
100	100	−	−	−	−	−	−								
95	92	−	−	−	−	−	−								
90	85	−	−	−	−	−	−								
80	73	−	−	−	−	−	−								
70	62	−	−	−	−	−	−								
60	52	−	−	−	−	−	−								
50	42	−	−	−	−	−	−								
40	33	−	−	−	−	−	−	−	−	−	−	−	−		
30	24	−	−	−	−		−	−	−	−	−	−	−	−	−
25	20						+	+	+	−	−	−	−	−	−
20	16						+	+	+	+	+	+	+	+	−
Dist. water (Control)							+	+	+	+	+	+	+	+	+

ethyl alcohol with sterile distilled water. The concentrations of the 70 and 40 per cent by volume solutions were verified by means of a pyknometer. The results of the tests are summarized in TABLES 1 to 6, in which a +

TABLE 3
THE EFFECT OF VARIOUS CONCENTRATIONS OF ETHYL ALCOHOL
TEST ORGANISM: *Escherichia coli*

Alcohol (C_2H_5OH)		*Exposure of test organism to germicide*											
% by vol.	*% by wt.*	*Seconds*					*Minutes*						
		10	20	30	40	50	1	2	3	4	5	10	15
100	100	−	−	−	−	−	−						
95	92	−	−	−	−	−	−						
90	85	−	−	−	−	−	−						
80	73	−	−	−	−	−	−						
70	62	−	−	−	−	−	−						
60	52	−	−	−	−	−	−						
50	42	−	−	−	−	−	−						
40	33	−	−	−	−	−	−						
30	24	+	+	+	−	−	−	−	−	−	−	−	−
25	20				+	+	+	+	−	−	−	−	−
20	16				+	+	+	+	+	+	+	+	−

TABLE 4
THE EFFECT OF VARIOUS CONCENTRATIONS OF ETHYL ALCOHOL

Alcohol (C_2H_5OH)		*Exposure of test organism to germicide:*																			
		Salmonella typhosa, H901													*Bacillus anthracis*						
% by vol.	*% by wt.*	*Seconds*					*Minutes*								*Hours*						
		10	20	30	40	50	1	2	3	4	5	10	15	30	2	24	48	72	120	144
100	100	−	−	−	−	−	−								+	+	−	−	−	−
95	92	−	−	−	−	−	−								+	+	−	−	−	−
90	85	−	−	−	−	−	−								+	+	−	−	−	−
80	73	−	−	−	−	−	−								+	+	−	−	−	−
70	62	−	−	−	−	−	−								+	+	−	−	−	−
60	52	−	−	−	−	−	−								+	+	−	−	−	−
50	42	−	−	−	−	−	−								+	+	−	−	−	−
40	33	−	−	−	−	−	−								+	+	+	−	−	−
30	24	+	+	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−
25	20						+	+	+	+	+	−	−	−	+	+	+	+	+	−
20	16						+	+	+	+	+	+	−	−	+	+	+	+	+	−

sign indicates growth of the test organisms, a − sign indicates no growth of the test organisms, and a ± sign indicates that, in some tests, the test organisms grew and, in other tests, the test organisms failed to grow in the subculture medium.

TABLE 5

THE EFFECT OF VARIOUS CONCENTRATIONS OF ETHYL ALCOHOL

TEST ORGANISM: *Staphylococcus aureus*

Alcohol (C_2H_5OH)		*Exposure of test organism to germicide*																
		Seconds					*Minutes*								*Hours*			
% by vol	*% by wt.*	10	20	30	40	50	1	2	3	4	5	10	15	45	1	1½	2	3
100	100	+	±	+	±	−	−	−	−	−	−	−	−					
95	92	−	−	−	−	−	−	−	−	−	−	−	−					
90	85	−	−	−	−	−	−	−	−	−	−	−	−					
80	73	−	−	−	−	−	−	−	−	−	−	−	−					
70	62	−	−	−	−	−	−	−	−	−	−	−	−					
60	52	−	−	−	−	−	−	−	−	−	−	−	−					
50	42	+	−	−	−	−	−	−	−	−	−	−	−					
40	33	+	+	+	+	+	+	+	+	±	−	−	−					
30	24										+	+	+	+	−	−	−	−
25	20										+	+	+	±	+	−	−	−
20	16										+	+	+	+	+	+	+	−
Dist. water (control)											+	+	+	+	+			

TABLE 6

THE EFFECT OF VARIOUS CONCENTRATIONS OF ETHYL ALCOHOL

TEST ORGANISM: *Streptococcus pyogenes* No. 1685

Alcohol (C_2H_5OH)		*Exposure of test organism to germicide*																
		Seconds					*Minutes*											
% by vol.	*% by wt.*	10	20	30	40	50	1	1½	2	3	3½	4	5	10	15	30	45	60
100	100	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−		
95	92	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		
90	85	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		
80	73	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		
70	62	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		
60	52	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−		
50	42	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−		
40	33	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−		
30	24						+	+	+	+	+	+	+	+	+	+	−	−
25	20						+	+	+	+	+	+	+	+	+	+	+	−
20	16						+	+	+	+	+	+	+	+	+	+	+	−

Discussion

For the sake of brevity, no extensive citations to the literature will be given. The literature on the germicidal action of alcohol appears to have been covered adequately by Price (1939). The more recent reports will be cited, however.

The results of various investigators testing the germicidal action of ethyl alcohol in recent years are in agreement with the findings herein reported, and are very different from the reports of earlier investigators who used bacteria dried on threads or garnets. Smith (1947) reported that a 95 per cent concentration was the only one between 30 and 100 per cent which in 15 seconds killed *Mycobacterium tuberculosis* suspended in sputum. After 30 seconds' exposure, concentrations of 50 to 100 per cent killed *M. tuberculosis*. Closely similar results were obtained when tubercle bacilli were suspended in water instead of sputum; 30 seconds' exposure of the bacilli to either 100 or 95 per cent destroyed the bacilli as judged by culture and animal tests. When sputum naturally infected with *M. tuberculosis* was dried on glass cover slips and then exposed to the action of the alcoholic solutions, the 50 and 70 per cent solutions were much more effective than the 95 and 100 per cent solutions. This is approaching the conditions and results of investigators of thirty to fifty or more years ago.

Robertson *et al.* (1948) have reported that after an exposure of 24 hours at 37°C., 5 per cent alcohol killed *Diplococcus pneumoniae*, type 1; 8 per cent killed a Group C hemolytic streptococcus; and 15 per cent killed *Micrococcus pyogenes*, variety *albus*. Molinas and Brewer (1949) found that a solution of 50 per cent alcohol and 10 per cent acetone, with occasional exceptions, gave equal or superior results to those obtained with commercial preparations intended for disinfecting the skin. These authors came to the conclusion that the activity of proprietary preparations designated for disinfecting the skin is largely dependent upon the alcoholic solution in which they are incorporated. Since 50 per cent alcohol killed the vegetative form of Gram-negative organisms in less than 10 seconds and the vegetative form of Gram-positive organisms in less than 90 seconds in our tests, it would be rather difficult to improve upon the germicidal action of a 50 per cent alcoholic solution on organisms in a moist environment by the addition of another ingredient.

In its action on filterable viruses, Cunningham (1948) found that the virus of Newcastle disease of chickens was destroyed by 95 and 70 per cent ethyl alcohol but not by 40 and 25 per cent solutions.

These results are comparable to those obtained by Harrington and Walker (1903) with moist bacteria on silk threads. These investigators also employed organisms dried on silk threads for 48 hours in the incubator. In general, they found that absolute and 95 per cent alcohol were ineffective in destroying dried vegetative cells of bacteria in 24 hours' exposure and that 50 to 60 per cent alcoholic solutions were the most effective. It is unfortunate that the results of alcohol with dried organisms are cited in the textbooks as typical of the germicidal action of alcohol.

Summary

Escherichia coli, *Salmonella typhosa*, and *Serratia marcescens* were killed in 10 seconds or less by concentrations of ethyl alcohol of 100 to 40 per cent by volume. *Pseudomonas aeruginosa* was killed in 10 seconds or less by concentrations of 100 to 30 per cent by volume. Fifty to 90 seconds were required to kill *Micrococcus pyogenes*, variety *aureus*, and *Streptococcus pyogenes*, respectively, by absolute ethyl alcohol. The organisms were killed in 10 seconds or less by concentrations of 95 to 60 per cent. Forty-

eight hours were required to kill a 24 hour-old broth culture of *Bacillus anthracis* by concentrations of alcohol of 100 to 95 per cent.

From the wide range of concentrations in which ethyl alcohol destroys the moist vegetative forms of bacteria, it appears that too much emphasis has been placed on the importance of diluting 95 per cent alcohol to a lower concentration. The statement that 95 per cent alcohol is practically worthless as a germicide needs to be qualified. It is not true when referring to the moist vegetative forms of bacteria.

Compounds in 50 per cent alcoholic solutions, which are recommended for disinfectants, probably owe their germicidal action in large part to the alcoholic solvent.

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EVALUATION OF MERCURIAL COMPOUNDS AS ANTISEPTICS

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Mercurial compounds have not enjoyed a peaceful career as antibacterial chemicals since their popularization as germicides over sixty years ago (Koch, 1881). As early as 1889, it was shown (Geppert, 1889) that mercurial action could be counteracted with ammonium sulfide to reveal the bacteriostatic nature of their antibacterial activity.

During the ensuing years, other workers, using various techniques, have also shown that antibacterial activity of mercurials is only slowly bactericidal and mainly bacteriostatic. This bacteriostasis is even nullified by the presence of many types of sulfur-containing compounds, including sulfides (Geppert, 1889) (Hunt, 1937), thioglycollate (Marshall, Gunnison, and Luxen, 1940), body fluids such as plasma (Johnson and Meleney, 1942), and other organic matter (Green and Birkeland, 1944).

In the past decade, interest in various *in vivo* types of tests has indicated that the mercurials are ineffective *in vivo* and may be more toxic for tissue cells than bacterial cells, as shown in mice (Nungester and Kempf, 1942) (Sarber, 1942) (Spaulding and Bondi, 1947), tissue culture (Salle and Catlin, 1947), and embryonic eggs (Witlin, 1942) (Green and Birkeland, 1944), and with leucocytes (Welch and Hunter, 1940).

A recent report (Morton, North and Engley, 1948) asserted that three representative mercurial compounds, Mercurochrome, Merthiolate, and Metaphen, in aqueous media, did not fulfill the requirements of an antiseptic in that they failed to render bacteria incapable of causing infection. The data with animal infection correlated directly with the test tube neutralization method, using thioglycollate.

The data reported here will further evaluate some of the representative inorganic and organic mercurial compounds as antiseptics and as antibacterial chemicals *per se*. Some of the so-called "mercurial" preparations contain, besides the mercurial, alcohol-acetone, cresol, ethylene glycol, or benzyl alcohol in concentrations which are bactericidal. To call such preparations "mercurials" seems to be stretching a point and breaks the spirit, if not the letter, of the rules of the Council on Pharmacy and Chemistry of the American Medical Association (New and Non-Official Remedies, 1948), which suggest naming a preparation according to its most active ingredient. Preparations of that type are called, and many times labeled, "germicides." If germicidal, they owe it to the vehicle and method of test and not to the mercurial.

The compounds tested are listed in TABLE 1, giving proprietary name, chemical name, and concentration of use. As chemicals, they appear to have only one characteristic in common and that is the presence of mercury in the molecule. Some of these compounds appear to have other chemical groupings (such as phenolic) which might have antibacterial activity in their own right. However, as shown later, these compounds function as a complete, mercury-substituted molecule.

The animal experiments described previously (Morton, North, and Engley, 1948) were extended in this work to include the other mercurial compounds listed here, and, to allay certain criticism, the organisms were exposed at 32° to 34° C. (skin temperature) to the mercurial, instead of at room temperature. After ten minutes' exposure of 1 ml. of a highly virulent streptococcus culture to 9 ml. of mercurial, nutrient dextrose broth, thioglycollate broth, and serum broth were inoculated, and mice injected intraperitoneally. The data in TABLE 2 reveal that none of the mercurial compounds in water solution in the concentration of use could completely destroy or alter the test culture to prevent infection of the mice by the streptococcus. The toxicity of the mercurials for mice will be discussed later in this paper.

TABLE 1
MERCURIALS TESTED

Name	*Manufacturer*	*Chemical Name*	*Conc of Use (Per cent)*
Merbak	Schieffelin	2-acetoxymercuri-4-diisobutyl phenol	0.1
Mercarbolide	Upjohn	orthohydroxyphenylmercuric chloride	0.1
Mercresin	Upjohn	orthohydroxyphenylmercuric chloride + secondary-amyl tricresols	0.1 0.1
Mercuric chloride*	—	mercuric chloride	0.1
Mercuric iodide*	—	mercuric iodide	0.1
Mercurochrome	H W D†	disodium 2,7-dibrom-4-hydroxymercurifluorescein	2.0
Mercurophen	Sharpe & Dohme	sodium oxymercury-o-nitrophenolate	0.1
Merodicein	H W D†	monohydroxymercuridiiodoresorcinsulfonphthalein	0.2
Merthiolate	Lilly	sodium ethyl mercuri thiosalicylate	0.1
Metaphen	Abbott	anhydride of 4-nitro-3-hydroxymercuri-ortho-cresol	0.2
Phenyl mercuric borate*	—	phenyl mercuric borate	0.1

* Non-proprietary.
† Hynson, Westcott & Dunning.

Results similar to those described previously at room temperature and here at 32° to 34° C. were obtained with Merthiolate at 20° C. and 37° C. (Powell, 1948). The data of another investigator working with Mercurochrome (Brewer, 1948) cannot be compared because of the lack of virulence of his test organism.

In many instances, rapid *in vitro* tests are necessary for qualitative or quantitative evaluation of antibacterial activity. Many laboratories are not able to do routine or extensive animal testing because of time, facilities, and expense. The results obtained with a paper disc assay method (Hoffman and Phillips, 1950) devised to evaluate phenolic compounds appeared to offer many advantages as a routine, rapid, quantitative technique for testing and comparing the antibacterial activity of mercurial compounds.

The technique is similar to the paper disc assay method for penicillin, using *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus #209*) as the test organism. The technique utilizes flat-bottom petri dishes (Corning #3162) and porous tops. Nutrient agar, in 15 ml. quantities, is placed in the dishes as a base, and 5 ml. of seed agar are overlayered after cooling. The chemical solutions (.08 ml.) are placed on paper discs on the agar surface and the plates incubated at 37°C. for sixteen hours. The diameters of the zones of inhibition are measured and compared.

The results with various mercurial compounds in aqueous solution are shown in TABLE 3. The zones are similar to those obtained in the paper disc assay technique with penicillin and other antibiotics. The data from experiments using the 20 ml. agar plate indicate the relative antibacterial activity of the mercurials tested at their concentration of use. It reveals the low order of activity of Merodicein and mercuric chloride in comparison to phenyl mercuric borate, Merthiolate, and other compounds. The results of these tests could not be compared with phenol coefficient values because of the wide variance in published values (McCulloch, 1945).

TABLE 2

COMPARISON OF *In Vivo* AND *In Vitro* METHODS OF EVALUATING MERCURIAL ANTISEPTIC ACTIVITY

Compound	*Conc. of Use (%) Mice*	*Mice Dead / Mice Injected**	*Dextrose Broth*	*Dextrose Broth + 0.1% thioglycollate*	*Dextrose Broth + 10% serum*
Phenyl mercuric borate	0.1	8/10	−	+	+
Merbak	0.1	9/10	−	+	+
Merthiolate	0.1	10/10	−	+	+
Mercurophen	0.1	9/10	−	+	+
Mercuric iodide	0.1	8/10	−	+	+
Mercarbolide	0.1	8/10	−	+	+
Merodicein	0.2	10/10	−	+	+
Metaphen	0.2	7/10	−	+	+
Mercurochrome	2.0	10/10	−	+	+
Mercuric chloride	0.1	10/10	−	+	+
Water		10/10	+	+	+

* Mice injected and tubes inoculated with 0.1 ml. of a 1:2 dilution of the Streptococcus — mercurial mixture after a ten minute exposure at 32–34° C. Streptococcus recovered from heart's blood at autopsy.
− no growth within seven days.
+ growth of test organism.

Of particular interest is the fact that the red color of Mercurochrome diffuses further than the inhibition zone. This point might be important when delineating and disinfecting the area of action in surgery with colored disinfecting solutions. The same effect was noted with the tincture of Merthiolate containing an eosin dye.

While usually using 20 ml. of agar, the test may be modified to increase the sensitivity by using the disc on 5 ml. of agar, a .01 ml. drop of the

TABLE 3

COMPARISON OF ANTIBACTERIAL ACTIVITY OF MERCURIAL ANTISEPTICS BY PAPER DISC ASSAY METHOD

(20 ML. PLATE)

Compound	*Dilution of Use (Per cent)*	*Nutrient Agar Inhibition Zone (in mm.)*
Phenyl mercuric borate	0.1	33
Mercresin	0.1	32
Mercarbolide	0.1	32
Merthiolate	0.1	32
Mercurophen	0.1	29
Mercuric iodide	0.1	28
Metaphen	0.2	25
Mercurochrome	2.0	24
Merbak	0.1	23
Merodicein	0.2	18
Mercuric chloride	0.1	18

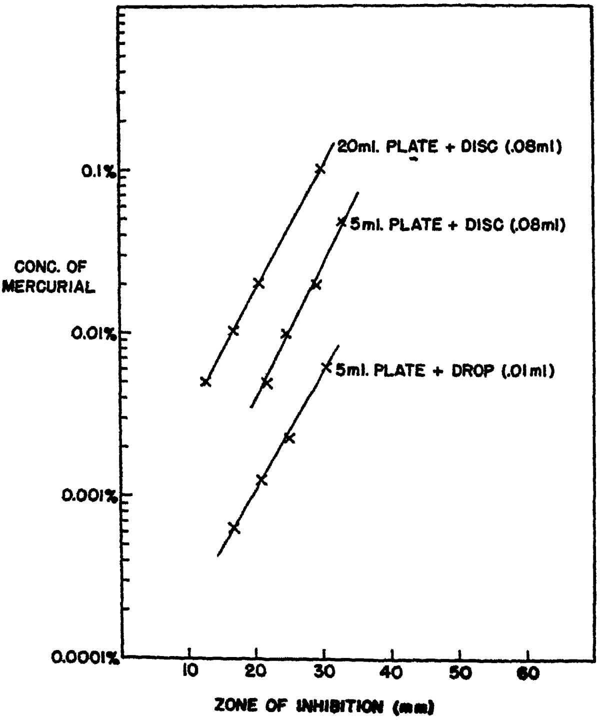

FIGURE 1. Comparison of plate method sensitivities.

chemical on the surface of the 5 ml. of agar, or both procedures combined. Similar modifications have been used with success in streptomycin assay (Forgacs and Kucera, 1946). FIGURE 1 points out that each modification increases the sensitivity approximately 5- to 10-fold. However, the increased sensitivity is not necessary for most routine testing.

A most important part of the paper disc technique is that it simulates skin open to the air. Tincture preparations which may show initial antibacterial activity on the skin have no residual effect in the rapid evaporation of the small amount of alcohol added to the paper disc. One might consider .08 ml. a small amount of test material, but it is close to the amount actually applied to the skin in any cut or abrasion. By actual test, we determined the amount applied per square inch of skin surface to be nearer 0.01 ml. This amount is 500 to 900 times less than the amount of solution tested in the liquid phenol coefficient type of test where the tincture vehicle is active in killing the organisms. The paper disc technique offers another advantage in that the pH effect in the liquid phenol coefficient test is lessened. Some of these mercurials have pH values at pH 9 to 10 or pH 3 to 4. One could expect such values to cause bacterial destruction in the test-tube *per se.*

TABLE 4

COMPARISON OF AQUEOUS AND TINCTURE MERCURIALS BY PLATE DISC ASSAY METHOD

Preparation	*Inhibition Zone (in mm.)*
Merthiolate, aqueous	30
Merthiolate, tincture	29.5
Merbak, aqueous	22
Merbak, tincture	22
Controls	
Alcohol (95%)	—
Alcohol 50%-acetone 10%	—
Water	—

The data in TABLE 4 reveal that the alcohol or alcohol-acetone mixture has no antibacterial activity and that, when two mercurials in both water and alcohol-acetone are tested, identical zones of inhibition for the aqueous and tincture preparations are found in the concentration of use. The controls with alcohol, alcohol and acetone, and water yielded no zone of inhibition. A similar picture is seen in FIGURE 2, where various concentrations of aqueous and tincture Merthiolate were compared. The tincture vehicle does not influence the zone of antibacterial action.

Because antibacterial activity upon dilution would be important in actual use, the mercurials were tested and compared for the effect of dilution on their activity. FIGURE 3 shows the resulting data in graph form. The compounds with curves beginning at the far right have greater initial activity. A steep slope in the curve indicates little loss of activity on dilution. The compounds at the left show little initial activity and a rapid decrease on dilution. Compounds such as phenyl mercuric borate, Mercarbolide, and Merthiolate show much greater activity by this test than Mercurochrome, Merbak, Merodicein, and mercuric chloride.

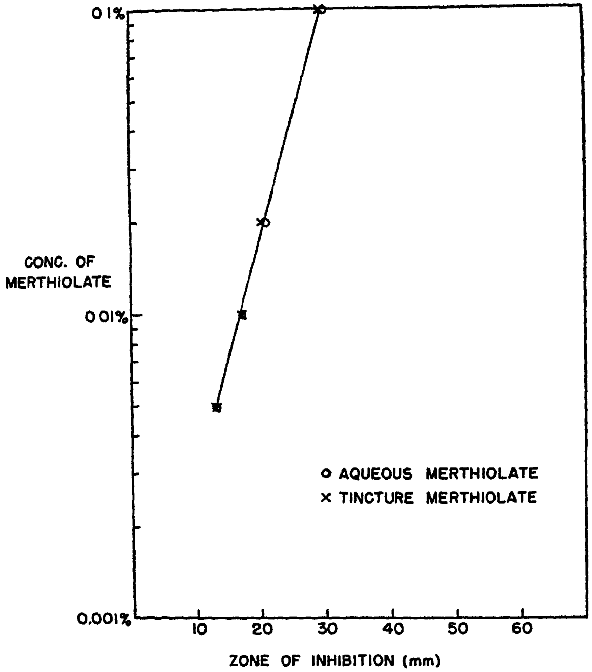

Figure 2. Comparison of aqueous and tincture Merthiolate (paper disc assay).

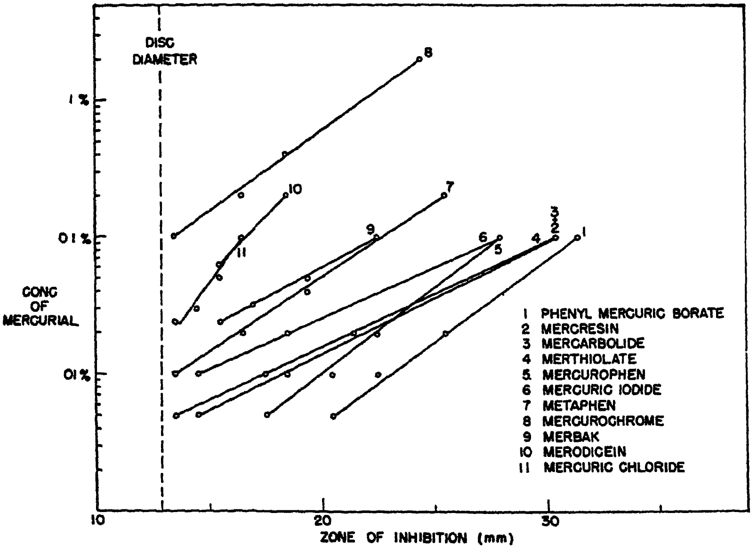

Figure 3. Effect of dilution on mercurial activity (paper disc assay).

Utilizing the LD_{50} values in mice and the least effective dilution by the disc assay method, it was possible to set up arbitrary toxicity or safety indices for the mercurials. These are shown in TABLE 5. The toxicity index was determined by the

$$\frac{\text{LD}_{50}\text{ dilution}}{\text{least effective dilution}}$$

and the safety index by comparing them conversely. The data indicate that mercuric chloride, Mercurochrome, and mercuric iodide have high toxicity values, while phenyl mercuric borate and Mercurophen have low toxicity indices by this method of comparison. These comparisons compare with other toxicity tests with leucocytes (Welch and Hunter, 1940), with tissue culture (Salle and Catlin, 1947), and with embryonic eggs (Witlin, 1942) (Green and Birkeland, 1944).

TABLE 5
COMPARISON OF MERCURIALS AS TO TOXICITY AND SAFETY INDICES

Compound	*Mercury* %	*Toxicity Index* $\frac{\textit{Toxic Dilution } (\text{LD}_{50})^*}{\textit{Effective Dilution}^\dagger}$	*Safety Index* $\frac{\textit{Effective Dilution}}{\textit{Toxic Dilution } (\text{LD}_{50})}$
Phenyl mercuric borate	59	.02	41.0
Mercurophen	43	.06	16.0
Merthiolate	49	.10	7.3
Mercarbolide	61	.20	4.7
Mercresin	—	.20	4.7
Merbak	44	.30	3.3
Metaphen	57	.51	1.9
Merodicein	24	.56	1.8
Mercuric iodide	44	.64	1.6
Mercurochrome	25	.68	1.4
Mercuric chloride	74	2.50	0.4

* LD_{50} for 20 g mice
† The greatest dilution showing least measurable inhibition by paper disc assay method

The paper disc assay method may be adapted for use with chemical neutralizers such as thioglycollate or with natural substances with which the chemicals would have to contend in the field of action, such as serum. The addition of these substances would determine whether the mercurials are actually killing the bacteria or merely preventing their multiplication in so-called bacteriostasis. The use of thioglycollate is not of just academic interest in test-tube studies, but actually simulates many sulfur-containing substances such as skin, perspiration, blood, serum, and tissue debris. TABLE 6 shows the results when the mercurials are tested in three media: nutrient, thioglycollate, and serum. While the mercurials show good activity in ordinary nutrient media, the thioglycollate and serum both neutralize the action, indicating that the mercurial compounds are not active in destroying bacteria in their presence. The question arises, then, as to how they could work *in vivo*, and the results in animal experiments have indicated that they are inactive.

The plate assay technique may be adapted for other types of testing similar to the method described in Bulletin 198 of the F.D.A. (Ruehle and Brewer, 1931). For instance, some of the adhesive bandages employ Mercurochrome on the bandage pad. These bandages were applied to the surface of the seeded agar plates similar to the paper discs under identical conditions of incubation to determine whether any inhibition of bacterial growth could be obtained. When the adhesive bandage was removed, it was found that the adhesive seemed to have as much or more antibacterial activity than the mercurial on the bandage pad. When tests were performed with thioglycollate or serum in the agar, even the minimal antibacterial activity of the mercurial was nullified. It was suggested that the adhesive was merely removing much surface growth, but this was shown not to be the case. The presence of any physical barrier on the surface of the agar would prevent some surface growth, but close observation revealed that the adhesive actually has antibacterial properties.

TABLE 6
COMPARISON OF ANTIBACTERIAL ACTIVITY OF MERCURIALS USING NEUTRALIZERS BY PAPER DISC METHOD

Compound	*Dilution of Use (%)*	*Nutrient Agar inhibition zone (in mm.)*	*Nutrient agar + 0.2% thioglycollate*	*50% Serum Agar*
Phenyl mercuric borate	0.1	33	—	—
Mercresin	0.1	32	—	—
Mercarbolide	0.1	32	—	—
Merthiolate	0.1	32	—	—
Mercurophen	0.1	29	—	—
Mercuric iodide	0.1	28	—	—
Metaphen	0.2	25	—	—
Mercurochrome	2.0	24	—	—
Merbak	0.1	23	—	—
Merodicein	0.2	18	—	—
Mercuric chloride	0.1	18	—	—

— = no inhibition zone.

A report commenting on the ineffectiveness of mercurials in sterilizing instruments (Brewer, 1939) suggested that organic solvents or wetting agents might increase their activity. It was reported, however, that thirty-nine surface reducing chemicals did not increase the bactericidal or bacteriostatic action of ten mercurials when no pH adjustments were made (Gershenfeld and Witlin, 1941). A recent published abstract (Lilley and Brewer, 1949) revealed that, while neither Mercurochrome nor sodium lauryl sulfate (Duponol), an anionic surface-active agent, could kill their test organism, a combination of the two could destroy the organism readily. A previous paper (Birkeland and Steinhaus, 1939) had reported that sodium lauryl sulfate was active only against Gram-positive organisms.

The paper disc assay method was applied to study the effect of surface active agents on mercurial activity. Three surface-active agents at a 1 per cent concentration were tested for their activity with Mercurochrome: (1) Roccal (cationic), (2) Duponol (anionic), and (3) Triton A20 (nonionic). The data are combined in TABLE 7. Duponol increased the activity of the mercurial slightly in nutrient agar and was found to be active alone in the presence of thioglycollate, but not in serum. Roccal precipitated the mercurial and reduced its activity, making the mixture impractical. Roccal appeared to be active in the presence of serum at the concentration tested. Triton A20 showed no activity of its own, but apparently increased the mercurial inhibition zone on nutrient agar. However, the mixture was not active in thioglycollate or serum. The surface-active agents were used with the mercurial at a lower (0.1 per cent) concentration with a markedly reduced effect. It is evident that while certain surface active agents might increase the apparent antibacterial action of mercurials in the nutrient agar tests, such activity is nullified by the presence of serum.

Preliminary experiments with other chemicals suggested that the paper disc assay method might be utilized in comparing the antibacterial activity of chemicals within various classes of compounds, such as halogens and surface active agents. The chemicals need not be water soluble for test, and may be in various vehicles, such as tinctures, ointments, or soaps or on various materials, such as paper or cloth.

TABLE 7
EFFECT OF SURFACE ACTIVE AGENTS ON MERCURIAL ACTIVITY DISC ASSAY METHOD
(20 ML. PLATE)

Mercurial	*Surface Active Agent*	*Medium*		
		Nutrient Agar	*+ Thioglycollate 1%*	*Serum Agar 50%*
		(Zone of Inhibition in mm.)		
Mercurochrome 1%	— — —	23	—	—
Mercurochrome 1%	+ Duponol 1%	25	26	—
	Duponol 1%	24	23	—
Mercurochrome 1%	+ Roccal 1%	20	17	16
	Roccal 1%	26	25	18
Mercurochrome 1%	+ Triton A20 1%	27	—	—
	Triton A20 1%	—	—	

Summary and Conclusions

(1) Animal tests with representative organic and inorganic mercurial compounds indicate that the mercurials are unable to prevent infection when virulent streptococci are exposed to mercurial action for ten minutes in the phenol coefficient type of test and then injected into mice. These findings correlate well with the thioglycollate or serum neutralizing culture medium tests.

(2) By means of the paper disc assay method, mercurial compounds which were found active in ordinary culture media were found to be inactive in the presence of thioglycollate and, especially important, were found to be inactive in the presence of serum. The technique has been found useful in elimination of the antibacterial action of the tincture vehicles, and in

differentiating between the activity of various ingredients of antiseptic preparations.

(3) A toxicity index and safety index comparing the mercurials as to their antibacterial activity and toxicity for mice has been found to correlate with other published toxicity indices determined in eggs, with leucocytes, and with tissue culture techniques.

(4) Adhesive bandage preparations containing a mercurial were found to have no antibacterial activity in the presence of serum.

(5) The paper disc technique has been recommended as a method for rapid evaluation of antibacterial activity of other antiseptic preparations.

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ASSAY OF ANTISEPTICS AT DIFFERENT TIMES AFTER APPLICATION TO HUMAN SKIN

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Although a vast amount of information has been published concerning the many chemicals intended as antiseptics for human use, it would appear to help very little in answering the final question of practical effectiveness when applied to the human skin. The present authors believe, from many years of experience, that the effectiveness of antiseptics as applied to the human body has never been established clearly, and that the proper specific laboratory tests to reflect degree of utility have not yet been devised. This situation would also seem to be implied by the fact that there exists a long and conglomerate list of tests set up to "prove" or "disprove" the usefulness of proposed antiseptics.

There is very little information as to what happens to antiseptics once they are applied to tissues. This is in sharp contrast, for example, to the available knowledge dealing with the fate of sulfonamides, antibiotics, *etc.* after use of these agents. Are the antiseptics fully as active as the result of tube tests indicate, or are they rapidly converted to an inactive form? In this report, we have attempted, through preliminary experiments on normal human skin, to learn something of the rate at which the activity of antiseptics is lost when they are painted on the skin.

Materials and Methods. In the tests as reported here, we have used the following antiseptics: (1) Tincture Merthiolate, 1:1000; (2) Tincture Metaphen, 1:200; (3) Tincture Zephiran, 1:1000; (4) Tincture Iodine, NF strong (half strength).

Without any preliminary treatment of the skin, 0.2 cc. quantities of the various antiseptics were applied evenly over square areas of skin measuring 2 inches on each side. Skin on the flexor area of the forearm was utilized, and 19 adult persons (8 males and 11 females) were included in the tests. These experiments were carried out during the summer. This point is mentioned in passing, but the possible effect of climatic factors is not taken into account in any interpretation of the results.

Following application to the skin, we attempted after various time intervals to recover the antiseptic from the skin to determine quantitatively the amount recovered, using stock antiseptic as a control. The different time intervals employed were 10 minutes, 1, 2, 4, and 8 hours. For recovering antiseptics from the skin, we used 5 cc. of solvent. By means of two pairs of small forceps and a single pledget of absorbent cotton, a small quantity of solvent was swabbed over the antiseptic area. The solvent was then soaked up as well as possible in the cotton and wrung out of the cotton into an empty vial. Further aliquots of the 5 cc. of solvent were similarly handled. In all, 8 to 10 consecutive swabbings of the antiseptic area were done. Each sample of collected washings was adjusted to 5 cc. with a little additional solvent. The solvents which we used were beef extract broth, 50 per cent alcohol, or 95 per cent alcohol. With this technic. if all the antiseptics were recovered from the skin, the washings would represent a 25-fold dilution of the stock antiseptic (*i.e.*, 0.2 cc. antiseptic diluted up to 5 cc.).

The different washings were processed to eliminate contaminating or residual organisms which might interfere with subsequent assay and to remove the alcohol from the alcohol washings. By use of comparably treated stock solutions of the antiseptics as controls, we found that: (1) Merthiolate-broth washings could be Seitz-filtered or heat-sterilized without loss in activity; (2) Merthiolate-alcohol washings could be Seitz-filtered and/or evaporated to dryness also without loss; (3) Metaphen-broth washings could be Seitz-filtered or heat-sterilized without loss in activity; (4) Metaphen-alcohol washings could be Seitz-filtered and/or evaporated to dryness also without much loss (however, these washings surprisingly contained only a trace of activity to begin with); (5) Zephiran-broth and alcohol washings lost approximately 90 per cent of activity in Seitz-filtration and could be heat-sterilized and/or evaporated to dryness without loss (however, the alcohol washings, and not the broth washings, contained the activity); (6) iodine-alcohol washings, but not broth washings, appeared active. Inconclusive tests have been done to estimate activity after the alcohol was eliminated. In other words, the activity (with alcohol not

TABLE 1
ANTISEPTIC ACTIVITY OF WASHINGS FROM THE SKIN

Solvent	*Merthiolate (filterable + heat-stable)**	*Metaphen (filterable + heat-stable)*	*Zephiran (nonfilterable + heat-stable)*	*Iodine (?)*
Beef extract broth	+	+	NIL	NIL
50% Alcohol	+	NIL	+	+
95% Alcohol	+	?	NIL	+

* Filterable—maintenance of activity after passage through a Seitz filter, heat-stable—maintenance of activity when reduced in volume partially or to dryness in salt water bath. Comparable control solutions were tested at the same time.

TABLE 2
TESTS OF ACTIVITY OF MERTHIOLATE ON THE SKIN

Time after application (hours)	*Solvent for washings*													
	Broth					*50% alcohol*				*95% alcohol*				
	Test subjects					*Test subjects*				*Test subjects*				
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
1	100	50	100	100	100	50	25	50	50	50	12	100	100	50
2	50	25	50	50	100	50	25	25	50	25	3	50	50	50
4	50	25	25	25	50	50	25	25	50	12	−3	25	50	−12
8	25	25	−6	12	50	25	6	12	25	12	−3	25	25	−12

Each figure indicates percentage of activity of control.

eliminated) was very close to the activity of alcohol alone. The foregoing 6 points are summarized in TABLE 1.

Final Assays of Washings. All washings prepared as we have indicated were reconstituted up to 5 cc. volumes with sterile beef extract broth preliminary to assay. At this point, a 25-fold increase in dilution of the stock antiseptic (in terms of full recovery) would have been attained.

Doubling dilutions of these washings were next made in sterile beef extract broth. Comparable stock antiseptic was similarly treated, and this included preliminary processing as indicated above. Two and one-half cc. volumes of these dilutions were seeded with 1 loopful of a 24-hour broth culture of *Staphylococcus aureus* 209. Presence or absence of growth was determined on the basis of turbidity after 16 hours' incubation. The titer of the washings has been expressed as the percentage of full titer obtained with treated control antiseptic. In general, by using a series of doubling dilutions, one could test for 100, 50, 25, 12, 6, and 3 per cent of full activity.

TABLE 3

TESTS OF ACTIVITY OF METAPHEN ON THE SKIN

Time after application (hours)	*Solvent for washings*										
	Broth							*50% Alcohol*			
	test subjects							*test subjects*			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
1	25	50	25	50	50	50	100	6	−6	−6	−6
2	25	50	25	25	0	0	0	0	0	0	6
4	25	25	25	25	0	0	0	0	0	0	−6
8	12	25	6	12	0	0	0	0	0	0	−6

Each figure indicates percentage of activity of control. "0" indicates that the test was not done.

TABLE 4

TESTS OF ACTIVITY OF ZEPHIRAN ON THE SKIN

Time after application	*Solvent for washings*							
	Broth				*50% Alcohol*			
	test subjects				*test subjects*			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
10 min.	0	0	0	0	50	50	25	25
1 hour	3	−3	−3	−3	25	12	6	6
2 hours	−3	−3	−3	−3	25	6	12	6
4 hours	−3	−3	−3	−3	12	6	6	3
8 hours	−3	−3	−3	−3	12	6	6	3

Each figure indicates percentage of activity of control. "0" indicates that the test was not done.

The results obtained with the 3 different solvents or diluents are set forth in TABLES 2, 3, and 4. It will be observed that Merthiolate was recovered from the skin fairly regularly with beef extract broth and also with alcohol. Metaphen was recovered better with beef extract broth than with alcohol. Zephiran, on the other hand, was recovered better with alcohol than with beef extract broth.

Both of the mercurials, therefore, are recoverable with aqueous solvent (broth), whereas Zephiran appears to be recoverable in substantial titer only when alcohol is used. In the main, the two mercurials persist longer in extractable form than does Zephiran. Swabbing for a longer time, of course, might lead to the recovery of more Zephiran.

We are unable to present any quantitative information on iodine. We were unable to recover very much iodine from skin applications by means of beef extract broth solvent. Alcohol, on the other hand, appeared to recover a good part of the iodine in the short tests. However, the diluent interfered with the assay determinations (as judged by the alcohol control), and we were unable to rid the solution readily of alcohol. Furthermore, iodine sublimed considerably on heating. We had little success in recovering iodine from the skin by use of potassium iodide solutions.

Summary

We have tested Merthiolate, Metaphen, Zephiran, and iodine for the property of remaining in inactive form after applications to human skin. Assays of washings from treated areas show a decrease of activity with the passage of time. The results suggest that Merthiolate is recoverable by aqueous or alcohol solvents, Metaphen by aqueous but not by alcohol, and Zephiran by alcohol but not by aqueous solvents. Our methods did not yield satisfactory results with iodine. It is concluded from these results that all the antiseptics remained *in situ* for as long as eight hours in titers stronger than those necessary for suppression of staphylococcal growth, since the washings had to be diluted at least 25- to 50-fold in order to obtain growth of the test organisms.

MERCURIALS AS ANTISEPTICS

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Before most of the common pathogens were isolated, it was a well-recognized fact that the use of mercurial salts would prevent sepsis. Compounds were clinically evaluated on their ability to prevent sepsis in open wounds and not on whether the compounds were germicides or bacteriostatic agents. Since these early physicians did not have laboratory facilities at their disposal, their reports in the literature describe the prevention of sepsis in large numbers of cases by the use of mercurials with hundreds of years of previously untreated infected cases acting as controls. Here is, indeed, the ideal antiseptic test—the ability of these compounds to act as antiseptics under actual conditions of use.

To suggest that the mercurials are not antiseptics simply because they are easily neutralized by substances found in the body, such as glutathione, is like suggesting that the sulfonamides are not satisfactory chemotherapeutic agents, since they are easily neutralized by *p*-aminobenzoic acid.

Although we are indebted to Koch[1] for much of our early progress in the field of medical bacteriology, he is probably more responsible than any other one person for the misconception of the true value of mercurial antiseptics. Immediately after his famous string experiments with anthrax spores, the medical world believed that it had in its hands an all-powerful germicide which would destroy any disease-producing organism, because, apparently, Koch had shown that very great dilution of mercury bichloride would kill the spores of the most resistant organisms. Since bichloride and several other mercurial salts were thought to be very potent germicides, they were suggested as agents for the sterilization of instruments and for use as germicides in various medical practices. Geppert,[2] in 1889, with carefully controlled experiments, showed that the inorganic mercurial salts were very poor germicides, although they possess an almost unbelievable antiseptic or, better, bacteriostatic ability.

At its best, mercury bichloride possesses several disadvantages which were known to Koch: it corrodes metal instruments; its apparent killing effect is diminished in the presence of organic matter; and it coagulates protein. Furthermore, mercury bichloride cannot be used on the skin over long periods of time without causing dermatitis. It is toxic when injected parenterally, and therefore cannot be used in chemotherapy. In spite of these objections and the observations of Geppert and others that mercury bichloride cannot be depended on to kill the spores of anthrax bacilli, it continued to be used because of its great antiseptic action. Chemists throughout the world attempted to produce a mercurial compound which would not have these disadvantages and perhaps might kill the sporulating organisms as well. The first products of these researches possessed few advantages over mercury bichloride, but, just at the close of World War I, several compounds appeared on the open market in the form of organic mercurials. Some of these were apparently free from the disadvantages of mercury bichloride. With certain types of laboratory tests, they seem to be more effective than mercury bichloride. Clinical results indicated that they could be relied on as antiseptics.

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Many claims have been made for the mercurials, some rightfully and some without the slightest scientific foundation. Among these claims, it was suggested that certain of the products could be used for the "cold sterilization" of surgical and dental instruments. In fact, several manufacturers placed on the market preparations especially designed for this purpose. Practically all the germicidal claims made for such products were based on phenol coefficient tests in which only *Micrococcus pyogenes* var. *aureus* and *Eberthella typhi* were used. No adequate tests were made with sporulating organisms such as *Bacillus anthracis*, *Clostridium tetani*, or the group of organisms causing gangrene.[3]

Claims of sterilization and of sporicidal activity found their way into many journals, textbooks, and standard references. Even as late as 1937, the Council on Pharmacy and Chemistry of the American Medical Association accepted the claim for one organic mercurial that it is "a potent germicide for the spore-bearing and non-spore-bearing bacteria."

In our efforts to disprove these claims of high germicidal and sporicidal activity, we as bacteriologists have employed all sorts of strong neutralizing agents, such as sodium thioglycolate and BAL. Rightfully, we have been very successful in convincing the medical profession, as well as the laity, that neither the inorganic nor the organic mercurials can be depended upon to act as germicides. It should be emphasized, however, that, while we have been concentrating upon the question of whether these compounds possess bactericidal or bacteriostatic activity, we have, for the most part, lost sight of the common sense purpose which Koch and the earlier workers had in mind for them; that is, to prevent sepsis under actual conditions of use.

Since we do not possess nontoxic neutralizers for most of the so-called germicides, we have not been equally strict in determining whether many of these act as antiseptics because they kill or simply because they inhibit growth.

The average person or physician purchases and uses the mercurial antiseptics to help prevent infection. With this practical use in mind, we have tried to produce infections in laboratory animals by various laceration methods. We have reported these experiments elsewhere, and it will suffice here to say that, since the controls which received the straight virulent cultures and no antiseptic would not be infected in many instances and never consistently, this type of testing has been abandoned in favor of other types of animal tests. Although we have been able to show about eighty per cent protection with mercurials in experiments in which many lethal doses of streptococci or pneumococci have been introduced into the peritoneal cavity of mice,[4] it is my feeling that this cavity does not represent a wound in which the mercurials should be used. It is, rather, the place to study the value of chemotherapeutic agents or antibiotics. In our efforts to approach more closely an actual condition in which the mercurials might be used, and since we could not consistently infect through skin abrasion, we have tried other routes of infection.

Since it is the purpose of this work to add to the information concerning the ability of the mercurials to prevent sepsis, we feel that several questions need to be answered. Could the mercurials prevent the germination of spores in the animal body? The criticism has arisen in the past, concerning streptococci and pneumococci, that they change in virulence and that differences in various works may be because of insufficient mouse passage. We wished to choose an organism that would obviate this difficulty, and since, with streptococci, pneumococci, and anthrax, death in the experimental animal is due to a generalized septicemia, we wished to employ an organism which would cause death if even a very small foci of infection developed. We have worked for many years with a strain of *Clostridium tetani* which is constant in its virulence and which always causes death in white mice, even though the foci of infection is so small as to escape detection. Spore suspensions of this organism were prepared and freed from vegetative cells by a previously described technique, and the stock suspension was made to contain about 1,000,000 spores per cc.[3]

The test, as we began the experiments, consisted of injecting a solution of calcium chloride as a necrotizing agent intramuscularly and following with a mixture of tetanus spores and antiseptic which were mixed just previously to being injected. The controls were run by simply omitting the antiseptic and substituting water or saline. As an example of this type of experiment, if one hundred mice are injected with 0.1 cc. of 6 per cent calcium chloride solution and then injected as nearly as possible in the same intramuscular site with tetanus and mercurial mixture, one gets about 80 per cent protection over a period of sixty days, whereas all the control animals will die of tetanus within four days. It should be emphasized at this time that the dilution of spores selected kills 100 per cent of mice within four days. By this method, if we had 100 MLD per injection, we could show consistent and repeatable protection.

In discussing these early experiments, Dr. Henry Welch suggested that, since the practice of mixing spores with the antiseptic in a test tube could be criticized as an *in vitro* test, the same experiment should be repeated injecting the spores first and then the antiseptics at various time intervals. Using this modification, very erratic results were obtained intramuscularly, since we could not hit the same muscular area each time. Therefore, we began the use of a bleb type test. The technique is to inject 0.1 cc. of tetanus spores diluted in 6 per cent calcium chloride subcutaneously in the backs of mice and follow at various time intervals by injecting 0.1 cc. of the market strength of antiseptic into the same bleb. By this simple technique, we were able to show very definite antiseptic activity (TABLE 1).

After confirming this experiment many times on several hundred animals, we were sufficiently confident of our technique to determine how much protection might be afforded by the more common bactericidal agents, such as iodine, phenol, *etc.*, by this method. Drug controls were run on the market strength of alkylbenzyldimethylammonium chloride, 2 per cent iodine (aqueous), and 2 per cent phenol.

Since our findings concerning the ability of the mercurials to protect against injection of spore formers were seemingly important to us and were in direct opposition to the common knowledge which has grown up in recent years concerning the mercurials, we felt it necessary to have confirmation. Knowing of the FDA's interest in newer techniques, we asked Dr. Slocum, Dr. Brewer, and Mr. Molinas of the FDA to confirm our findings.

TABLE 1

ANTISEPTIC ACTIVITY OF MERCUROCHROME ON *Clostridium tetani* SPORES*

(*Clostridium tetani* spores #7 diluted 1:50 in 6 per cent $CaCl_2$, injected subcutaneously into 60 mice, and followed immediately with 01. cc of 2 per cent aqueous Mercurochrome into the same bleb. Ten of the mice, used as controls, received water.)

No. of deaths		*Days*	*% Protected*
Treated (50)	2	7	96
	3	14	94
	7	25	86
	9	32	82
Controls (10)	10	3	0

* Mercurochrome is the Hynson, Westcott and Dunning brand of Merbromin. N.F.

TABLE 2

COMPARISON OF RESULTS OF TETANUS ANTISEPTIC EXPERIMENTS

Antiseptic	*F. D. A.**		*H. W. & D.*†		*Combined Results*	
	No. of deaths	*% of deaths*	*No. of deaths*	*% of deaths*	*No. of deaths*	*% of protection*
Alkylbenzyldimethyl-ammonium chloride	5	20	3	12	8	84
Mercurochrome.	5	20	4	16	9	82
Iodine 2% (aqueous)	6	24	4	16	10	80
Nitromersol-N. F.	8	30	7	28	15	70
Phenol 2% .	11	44	12	48	23	64
Sodium ethylmercurithiosalicylate.	16	64	11	45	27	46
Control...	25	100	25	100	50	—

* By S Molinas.
† By B. McLaughlin.

The experiments were run in duplicate by us and by the FDA, with the results shown in TABLE 2.

One might suggest that, although this test is wholly an *in vivo* one, the bleb serves only as a sac and that, in reality, since the antiseptic is injected immediately, the spores are held static. To offset this criticism, the experiment has been extended for various time periods to allow the phagocytes, lymph, or serum and the necrotizing agent to start work. Also, it is felt that this will more closely simulate what actually happens in the use of mercurials. One rarely applies an antiseptic immediately, and we were curious to know if the application of the antiseptic was of any value after the spores had had a chance to become imbedded in the wound The results of this type of experiment are shown in TABLE 3.

There appears to be little or no difference when the drug is given immediately or sixty minutes later. The lack of smoothness of the death curve could be explained only by the inadequacy of being perfectly accurate in injecting the drug at the same site each time.

It should be kept in mind that these experiments were made only in an effort to add to the knowledge of mercurial antiseptics. We are not suggesting for one moment that any antiseptic be used in lieu of antitoxin or that this is a standard technique which could be used for determining antiseptic activity.

In addition to these experiments, we wish to call attention to our work with Drs. Klein and Perez[5] of the University of Pennsylvania, in which we have shown that the mercurials will inactivate influenza virus *in vivo*. They will also inactivate bacteriophage, a process which is only static, however, since they can be reactivated by the use of BAL.

TABLE 3

ACTIVITY OF MERCUROCHROME INJECTED AT INTERVALS AFTER SPORE INJECTION

(*Clostridium tetani* spores #7 diluted 1:50 in 6 per cent $CaCl_2$, injected subcutaneously into 50 mice, and followed immediately and at regular intervals with 0.1 cc. of 2 per cent aqueous Mercurochrome into the same bleb. The treated mice were divided into 4 groups of 10 mice each; the remaining 10, used as controls, received water)

Interval after spore injection	*No. of mice*	*Time of 1st death (days)*	*No. of deaths in 30 days*	*% Protected*
Immediately	10	12	2	80
15 minutes	10	7	4	60
30 minutes	10	2	2	80
60 minutes	10	5	4	60
		No. of deaths	*Time (days)*	
Controls	10	10	3	0

The very high bacteriostatic property of these compounds as compared with the relatively low bacteriostatic ability of the more common germicides has led to many attempts to combine various types of these compounds in order to produce a more satisfactory local antiseptic for general use. Our efforts in this field have recently been reported.[6] Twenty-four commercially available wetting agents have been tested in combination with the various mercurials. Several of these agents were discarded because of tissue toxicity, because they are neutralized by serum, or because they were incompatible with the mercurial being studied. We have found one of the anionic surface active agents, sodium lauryl sulfate, a paraffin chain salt, the most generally satisfactory of all the compounds studied. For the purpose of this paper, we shall limit our discussion to this one compound. We have chosen organisms which might be found on the skin and in minor cuts and abrasions, and in addition, we have included some organisms which have been reported to be very resistant to the mercurials in general. The commercially available material must be purified before it is satisfactory for use. Unless this is done, the material will precipitate out in the cold and render the solutions turbid.

No spore-forming organisms have been included, since we have reported elsewhere[4] that spores will remain viable even though they are suspended in the market strength of the mercurials for over twelve years. None of the surface-active agents available at the present time has altered this finding.

We have used, at their recommended market strength, those mercurials listed in NNR, USP, or NF. They have been used in aqueous solution, since we did not wish to complicate this study with the additive effect of the tincture solvents. None of these mercurials can be depended upon to act as germicides if reducing agents are employed as neutralizers. In our tests, we have used the sodium lauryl sulfate in 0.1 per cent concentration, This produces a surface tension of 32 dynes at 25° C. At this concentration. the surface active agent is not bactericidal, except for some of the streptococci and pneumococci.

The description of the technique employed in this experiment is as follows. One cc. of a twenty-four hour broth culture of the test organism was added to 9 cc. aqueous mercurial; 1.0 cc. of the same twenty-four hour culture was also transferred to 9 cc. wetting agent in the proper dilution; and still another tube, containing 9 cc. mercurial, to which 0.1 per cent sodium lauryl sulfate had been added, was inoculated with 1.0 cc. of this culture. All three tubes were allowed to stand at room temperature for various time intervals: ten minutes, thirty minutes, one hour, and two hours. One-tenth cc. from each tube was subcultured into 15 cc. standard fluid thioglycolate medium. The subcultures were incubated at 37° C. and growth results recorded after twenty-four hours, forty-eight hours, and seven days. This procedure was followed for each of the six mercurials tested: 1:50 Mercurochrome, 1:500 Nitromersol, 1:500 Merodicein, 1:1,000 sodium ethylmercuri-thiosalicylate, 1:1,000 bichloride of mercury, and 1:1,500 phenyl mercuric nitrate. The organisms were all tested in combination with these mercurials and each protocol was repeated three times.

In our preliminary work, only 4 mm. loop subcultures were made. At the suggestion of Dr. Welch, however, larger quantities of mercurial-coated organisms were carried to the subculturing medium. We soon found that 0.1 cc. was the largest amount of mercurial that sodium thioglycolate in the standard medium could neutralize. Varying concentrations of the reducing agents, sodium formaldehyde sulfoxalate, mercaptoethanol, and BAL, were tried in the broth medium, but these were no more effective than the 0.1 per cent sodium thioglycolate which had previously been employed. It was then decided that the regular fluid thioglycolate medium would serve our purpose of determining the bactericidal properties of the tested mercurials.

TABLES 4-9 illustrate the seven-day results of subcultures into media

TABLE 4

BACTERICIDAL ACTIVITY OF MERCUROCHROME WITH 0.1 PER CENT SODIUM LAURYL SULFATE

(0.1 cc. subcultures into 15 cc. fluid thioglycolate medium and incubated 7 days.)

Solutions tested	*Micrococcus pyogenes var. aureus 209*				*Escherichia coli*				*Pseudomonas aeruginosa*			
	10 min.	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*
0.1 % Sodium lauryl sulfate	#	#	#	#	#	#	#	#	#	#	#	#
1:50 Aqueous Mercurochrome	#	#	#	#	#	#	#	#	#	#	#	#
1:50 Aqueous Mercurochrome & 0.1 % sodium lauryl sulfate	#	—	—	—	#	—	—	—	#	#	#	—

TABLE 5

BACTERICIDAL ACTIVITY OF PHENYL MERCURIC NITRATE WITH 0.1 PER CENT SODIUM LAURYL SULFATE

(0.1 cc. subcultures in 15 cc. fluid thioglycolate medium and incubated 7 days.)

Solutions tested	*Micrococcus Pyogenes var. aureus 209*				*Escherichia coli*				*Pseudomonas aeruginosa*			
	10 min.	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*
0.1 % Sodium lauryl sulfate	#	#	#	#	#	#	#	#	#	#	#	#
1:1,500 Phenyl Mercuric nitrate	#	#	#	#	#	#	#	#	#	#	#	—
1:1,500 Phenyl Mercuric nitrate & 0.1 % sodium lauryl sulfate	#	—	—	—	#	—	—	—	#	—	—	—

TABLE 6

BACTERICIDAL ACTIVITY OF MERODICEIN WITH 0.1 PER CENT SODIUM LAURYL SULFATE*

(0.1 cc. subcultures in 15 cc. fluid thioglycolate medium and incubated 7 days.)

Solutions tested	*Micrococcus pyogenes var. aureus 209*				*Escherichia coli*				*Pseudomonas aeruginosa*			
	10 min.	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*
0.1 % Sodium lauryl sulfate	#	#	#	#	#	#	#	#	#	#	#	#
1:500 Aqueous Merodicein	#	#	#	#	#	#	#	#	#	#	#	#
1:500 Aqueous Merodicein & 0.1 % sodium lauryl sulfate	—	—	—	—	#	#	—	—	#	#	—	—

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* Merodicein is the Hynson, Westcott and Dunning brand of monohydroxy-mercuridiiodoresorcinsulfonphalein-sodium.

TABLE 7

BACTERICIDAL ACTIVITY OF SODIUM ETHYLMERCURI-THIOSALICYLATE WITH 0.1 PER CENT SODIUM LAURYL SULFATE

(0.1 cc. subcultures in 15 cc. fluid thioglycolate medium and incubated 7 days)

Solutions tested	*Micrococcus pyogenes var. aureus 209*				*Escherichia coli*				*Psuedomonas aeruginosa*			
	10 min.	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min*	*1 hr.*	*2 hrs*	*10 min*	*30 min*	*1 hr.*	*2 hrs*
0.1% Sodium lauryl sulfate	#	#	#	#	#	#	#	#	#	#	#	#
1:1,000 Sodium ethylmercuri-thiosalicylate	#	#	#	#	#	#	#	#	#	#	#	#
1:1,000 Sodium ethmercuri-thiosalicylate & 0.1% sodium lauryl sulfate	—	—	—	—	#	—	—	—	#	#	—	—

TABLE 8

BACTERICIDAL ACTIVITY OF BICHLORIDE OF MERCURY WITH SODIUM LAURYL SULFATE

(0.1 cc. subcultures in 15 cc fluid thioglycolate medium and incubated 7 days)

Solutions tested	*Micrococcus pyogenes var. aureus 209*				*Escherichia coli*				*Pseudomonas aeruginosa*			
	10 min	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*
.1% Sodium lauryl sulfate	#	#	#	#	#	#	#	#	#	#	#	#
:1,000 Bichloride of of mercury	#	#	#	#	#	#	#	#	#	#	#	#
.:1,000 Bichloride of mercury & 0.1% sodium lauryl sulfate	#	—	—	—	#	—	—	—	#	#	—	—

TABLE 9

BACTERICIDAL ACTIVITY OF NITROMERSOL WITH SODIUM LAURYL SULFATE

(0.1 cc. subcultures in 15 cc. fluid thioglycolate medium and incubated 7 days)

Solutions tested	*Micrococcus pyogenes var. aureus 209*				*Escherichia coli*				*Pseudomonas aeruginosa*			
	10 min.	*30 min*	*1 hr.*	*2 hrs.*	*10 min*	*30 min.*	*1 hr.*	*2 hrs.*	*10 min.*	*30 min.*	*1 hr.*	*2 hrs.*
0.1% Sodium lauryl sulfate	#	#	#	#	#	#	#	#	#	#	#	#
1:500 Nitromersol	#	#	#	#	#	#	—	—	#	—	—	—
1:500 Nitromersol & 0.1% sodium lauryl sulfate	—	—	—	—	#	—	—	—	#	—	—	—

containing reducing agents of three representative organisms tested against the six mercurials with and without addition of sodium lauryl sulfate. In these tables, it will be noted that the mercurial alone was incapable of killing all of the organisms, as was the wetting agent in 0.1 per cent concentration, although a combination of the two was actually germicidal for each organism.

We claim no originality for the synergistic activity described. We wished only to use sufficient neutralizers and time limits to convince ourselves that this antibacterial activity was truly bactericidal and not only an extension of bacteriostasis. We realize, of course, that these synergistic tests in no way simulate actual conditions of use. Again, the experiments were performed only to add to our information and understanding concerning the mercurials.

We have tried to emphasize the fact that the mercurials were first used as antiseptics because they prevented sepsis. We have presented evidence to show that some of the mercurials are just as capable of preventing infection with *Clostridium tetani* spores as are the common germicides, even though they are applied one hour after the injection of the organisms. We have also presented data to show that, if one wishes to combine the mercurials with surface tension depressants, one will get greater germicidal activity than with either agent alone.

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